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Global approach (Part A): Are substances more persistent than test systems lead to believe? Non-extractable residues: experimental examination of suitable extraction methods in view of a long-term risk for the environment

Final Report

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Global approach (Part A): Are substances more persistent than test systems lead to believe? Non-extractable residues: experimental examination of suitable extraction methods in view of a long-term risk for the environment

Final Report

by

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Abstract

After exposure of soils and sediments to anthropogenic organic substances non-extractable residues (NER) can be formed. The proportion of a compound which remains non-extractable is strongly variable with the extraction procedure used, besides dependencies on substance properties and soil characteristics. In environmental studies degradation/dissipation times (DT-values) are influenced directly by the extraction method, as a more intense extraction procedure may release higher proportions of a parent compound and its transformation products, resulting in increased DT_{50}-values and a higher persistence. This may be of consequence for the environmental risk assessment of a compound.

However, in German and EU legal regulations, there is no exact and general definition, how to determine and characterise NER. Consequently, the comparability of NER data is limited. This could have particular influence on the evaluation of substances under different legal regulations (e.g. REACH chemicals, pesticides, biocides, pharmaceuticals).

In the past, NER have been widely neglected for persistence assessment as only the DT_{50} values (primary degradation) of parent compound and transformation products in soil and water/sediment systems (OECD guideline 307, 308, 309) were considered.

The ECHA PBT-guidance R.11 (2017) highlights the importance of the NER for assessment of persistence when testing transformation in water/sediment or soil systems (ECHA, June 2017). For this, further information on type and amount of NER are required. A harmonised concept to consider potentially remobilisable NER in the framework of persistence assessment (e.g. PBT, vPvB, POP) is needed.

Hence, the task of this study was to verify the sequential extraction scheme for the characterisation of non-extractable residues by Eschenbach and Oing (Eschenbach and Oing, 2013b). Furthermore, a standardised approach for the determination of non-extractable residues was developed, considering recent scientific advances (Schaeffer et al., 2018) to provide comparable NER data for the environmental risk assessment of organic substances.

To this end, extraction efficiencies and their variability were determined for 42 non-labelled organic chemicals spiked onto three soils applying a number of extraction techniques and conditions, developing an extraction procedure which provides high extraction efficiencies and a low variability for a broad spectrum of analytes.

Additionally, NER generated within soil transformation studies according to OECD TG 307 with ^{14}C-triclosan, ^{14}C-fenoxycarb and ^{14}C-acetaminophen and three standard soils (Lufa 2.2, Lufa 2.3 und Lufa 2.4) were analysed using sequential batch extraction and pressurised liquid extraction (PLE).

The widely universally applicable extraction procedure using PLE developed in this project is recommended for transformation studies in soil and water/sediment-systems to improve the comparability of the NER data and limit overestimation of NER.

Kurzbeschreibung

Nach der Exposition von Böden und Sedimenten mit organischen Substanzen anthropogenen Ursprungs können nicht extrahierbare Rückstände (NER) gebildet werden. Der Anteil einer Substanz, welcher als nicht extrahierbar im Boden zurückbleibt, hängt neben den Substanzeigenschaften und den Bodencharakteristika, stark vom angewendeten Extraktionsverfahren ab. In Studien zum Umweltverhalten von organischen Substanzen werden Abbau-/Dissipationszeiten (DT-Werte) direkt von der Extraktionsmethode beeinflusst, da ein intensiveres Extraktionsverfahren höhere Anteile dieser Stoffe und von deren Transformationsprodukten freisetzen kann, was zu erhöhten DT_{50}-Werten, also einer höheren Persistenz führt. Dies kann daher für die Umweltrisikobewertung von Stoffen relevant sein.

In der deutschen und EU-weiten Stoffregulierung gibt es kein standardisiertes Verfahren für die Bestimmung und Charakterisierung der NER. Folglich ist die Vergleichbarkeit vorhandener Daten zu NER limitiert. Dies dürfte besonderen Einfluss auf die Bewertung von Substanzen in unterschiedlichen regulatorischen Kontexten (z. B. REACH-Chemikalien, Pestizide, Biozide, Arzneimittel) haben.

Bei der Persistenzbewertung wurden die NER in der Vergangenheit weitgehend ignoriert, da nur die DT_{50}-Werte für den Primärabbau von Ausgangsverbindungen und deren Transformationsprodukten in Boden- und Wasser/Sediment- Systemen (OECD Guideline 307, 308, 309) berücksichtigt wurden.

In der PBT-Guidance R.11 der ECHA (2017) wird die Bedeutung der NER aus Transformationsstudien in Boden- bzw. Wasser/Sediment-Systemen für die Persistenzbewertung betont (ECHA, June 2017). Es werden deshalb weitergehende Informationen zu Art und Menge der NER benötigt. Für die Berücksichtigung von potentiell remobilisierbaren NER im Rahmen der Persistenzbewertung (z.B. PBT, vPvB, POP) wird ein harmonisiertes Konzept gebraucht.

Das Ziel dieser Studie war es, das sequenzielle Extraktionsschema zur Charakterisierung von nichtextrahierbaren Rückständen von Eschenbach und Oing (Eschenbach and Oing, 2013b) zu überprüfen. Weiterhin wurde ein standardisierter Ansatz zur Bestimmung von NER entwickelt, welcher vergleichbare NER Daten für die Umweltbewertung von organischen Substanzen liefert und dabei aktuelle wissenschaftliche Entwicklungen berücksichtigt (Schäffer et al., 2018).

Dazu wurden 42 nicht-markierte organische Substanzen auf drei unterschiedliche Böden dotiert und mit verschiedenen Extraktionsverfahren und -bedingungen extrahiert, um ein Extraktionsverfahren zu entwickeln, welches hohe Extraktionseffizienzen bei geringen Varianzen für ein breites Spektrum organischer Substanzen ermöglicht.

Weiterhin wurden Bodentransformationsstudien angelehnt an die OECD Richtlinie 307 mit ^{14}C-Triclosan, ^{14}C-Fenoxycarb und ^{14}C-Acetaminophen (Paracetamol) und drei Standardböden (Lufa 2.2, Lufa 2.3 und Lufa 2.4) durchgeführt. Die nicht extrahierbaren Anteile wurde nach sequentieller Schüttelextraktion und beschleunigter Lösemittelextraktion (PLE) quantifiziert.

Es wird empfohlen, das in diesem Projekt entwickelte und weitgehend universell einsetzbare PLE-Extraktionsverfahren bei Transformationsstudien in Boden- und Wasser/Sediment-Systemen einzusetzen, um die Vergleichbarkeit von NER-Daten zu verbessern und die methodische Überschätzung der NER somit zu minimieren.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C	Degree Celsius
µL	Microliter
^{14}C	Radioactive carbon isotope
$^{14}CO_2$	Radiolabelled carbon dioxide
3SBE	Three-step batch extraction
ACT	Acetaminophen
AEE	Apparent extraction efficiency
ASE	Accelerated solvent extraction (synonymous to PLE)
BfG	Bundesanstalt für Gewässerkunde (Federal Institute of Hydrology)
bioNER	Biogenic non-extractable residues
c	Concentration
c_0	Concentration at test start (day zero)
$CaCl_2$	Calcium chloride
CAS	Chemical Abstracts Service
CEC	Cation-exchange capacity
C_{org}	Organic carbon content
d	Day(s)
Da	Dalton
DFOP	Double First-Order in Parallel (model used for kinetic fitting, bi-phasic kinetics)
$DT_{50/90}$	Time in days for 50/90 % disappearance of the initially applied test substance
e.g.	For example (Latin: exempli gratia)
ECHA	European Chemicals Agency
EDTA	Ethylenediaminetetraacetic acid
EF	Extractable fractions
EFSA	European Food Safety Authority
EPI	Estimation Programme Interface
FA	Formic acid
FEC	Fenoxycarb
FEC-OH	Hydroxy-fenoxycarb
FOMC	First-Order Multi-Compartment (model used for kinetic fitting)
g	Gram
GC	Gas chromatography
GLP	Good laboratory practice
h	Hours

HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HS	Hockey Stick (model used for kinetic fitting, bi-phasic kinetics)
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
K_{oc}	Soil/water distribution coefficient normalized on the organic carbon content in soil
K_{ow}	*n*-octanol/water partition coefficient
LC	Liquid chromatography
logP	Logarithmic partition coefficient
LSC	Liquid scintillation counting
LUFA	Landwirtschaftliche Untersuchungs- und Forschungsanstalt
M	Molar [mol L^{-1}]
meq	Milliequivalent
Me-TCS	Methyl-triclosan
min	Minutes
mL	Millilitre
MS	Mass spectrometer
N	Nitrogen
NER	Non-extractable residues
ng	Nano gram
OECD	Organisation for Economic Co-operation and Development
pK_a	Acid dissociation constant
PLE	Pressurized liquid extraction
PLE_NER	Non-extractable residues remaining after pressurized liquid extraction
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
rpm	Rotation per minute
SAAEE	Soil averaged apparent extraction efficiency
SFO	Single First Order (simple one-compartment model used for kinetic fitting)
TCS	Triclosan
TMCS	Trimethylchlorosilane
TP	Transformation product
UBA	Umweltbundesamt (German Environment Agency)
US EPA	United States Environmental Protection Agency
WHC_{max}	Maximum water holding capacity

Summary

Organic chemicals are extensively introduced into the environment via numerous pathways. Agricultural products, e.g. pesticides and herbicides, are directly applied onto soils while biocides, detergents, human and veterinary pharmaceuticals and personal care products enter the environment after usage mainly via wastewater, digested sludge or manure.

While the amount of a compound in an environmental compartment is mostly observed to decrease with time, it rarely disappears entirely. Many chemicals can be immobilised or sequestered forming non-extractable residues (NER) in soil and sediment. It is estimated that about one third of the 4 million tons of pesticides (active ingredients) annually applied worldwide remain in the agricultural soils as NER (Barriuso et al., 2008; Food and Agriculture Organization of the United Nations (FAO), 2019). The quantitative proportion of a compound which remains non-extractable is operationally defined by the extraction procedure employed. It is influenced by experimental/environmental parameters such as incubation conditions, matrices and compounds investigated (Barriuso et al., 2008; Gevao et al., 2003; Loos et al., 2012; Mordaunt et al., 2005; Northcott and Jones, 2000b)

Although huge quantities of NER of anthropogenic chemicals remain in the soil, in Germany and the EU there is no common agreement, on how to determine and how to assess data on the non-extractable residues of chemicals. This is especially of relevance when different legal regulations apply, e.g. for industrial chemicals (REACH), plant protection products, pharmaceuticals and biocides.

Design of the study

The aim of this project was the verification of a sequential extraction scheme for the characterisation of non-extractable residues by Eschenbach and Oing (Eschenbach and Oing, 2013b) and the development of a standardised approach for the determination of non-extractable residues, providing data for the environmental risk assessment of chemicals.

To this end an extensive comparison of extraction procedures was conducted before analysing NER of three ^{14}C-labelled organic compounds (triclosan, fenoxycarb and acetaminophen) formed within soil transformation studies according to OECD guideline 307. In all experiments the standard soils Lufa 2.2, Lufa 2.3 and Lufa 2.4 were used, covering a range of different soil textures.

Comparison of soil extraction procedures

For the comparison of the extraction procedures, 42 organic chemicals with widely differing physicochemical properties and fields of application were spiked onto the set of three soils, incubated for 9 d and extracted before analysis by LC-MS/MS.

The results of this study highlight, that pressurised liquid extraction (PLE) at 100°C and 100 bar using a ternary extraction agent consisting of methanol, acetone and water (50/25/25, v/v/v) provided elevated extraction efficiencies and an acceptable uncertainty. It should be noted that using this method the extraction results were widely independent of the compounds spiked and the soil types selected. Consequently, this extraction method could be useful for the analysis of many organic substances in soil, in the context of substance registration.

It cannot be excluded that for individual compounds the extraction efficiency might be too low due to individual compound properties (e.g. thermolability). Thus, the applicability of any method to be applied needs to be confirmed for each individual compound (including TPs) and the matrix to be analysed. However, the PLE conditions mentioned above are at least appropriate as a good starting point for further method developments.

Soil transformation studies and characterisation of NER

Subsequently soil transformation tests with ^{14}C-triclosan, ^{14}C-fenoxycarb and ^{14}C-acetaminophen were conducted as radiotracer experiments according to OECD guideline 307 applying the set of 3 standard soils to generate non-extractable residues for further characterisation.

Triclosan, fenoxycarb and acetaminophen were selected as test substances, as these showed an extensive formation of NER and represent different substance groups from various fields of application.

Besides a characterisation of the fate of the compounds during incubation (triclosan and fenoxycarb 100 d, acetaminophen 35 d) the NER formed was characterised, using sequential batch extraction, PLE, silylation, EDTA extraction and HCl-treatment.

In all transformation experiments, the radioactivity applied was generally recovered quantitatively and in all cases volatile species, which were trapped in the paraffin traps, amounted to <1 % of radioactivity applied and were thus negligible.

For triclosan, between 11 % and 27 % of the applied radioactivity were mineralised to $^{14}CO_2$ within 100d. A three-step batch extraction (3SBE) with consecutive extractions with 0.01 M $CaCl_2$, methanol/water (50/50, v/v), methanol/acetone (50/50, v/v) was used to quantify the extractable fraction (EF) and non-extractable fraction (NER) in the incubated soil. The proportions of NER increased continuously with incubation time, while the radioactivity in the extractable fractions (EF) decreased for all soils. In Lufa 2.2 the EF decreased from 84 % after 7 d to 64 % after 100 d, while NER increased in the same time from 16 % to 34 % of applied radioactivity. However, in Lufa 2.3 a significantly higher percentage of NER (56 %) was formed. Chemical analysis was performed by radio-HPLC showing that triclosan was transformed into the transformation product (TP) methyl-triclosan (Me-TCS). The calculated DT_{50}-values for triclosan ranged between 2.3 and 56 d and DT_{90}-values between 19 d and 185 d.

In Lufa 2.2 and Lufa 2.3, the fraction of Me-TCS rose up to 22 % and 16 % of the applied radioactivity within 100 d. In contrast, Me-TCS peaked after 34 d of incubation at 60 % in Lufa 2.4 and decreased to 34 % after 100 d. The half-life of triclosan is strongly dependent on the extraction procedure used, since triclosan shows a very strong sorption towards soil. PLE was able to release additional radioactivity from the pre-extracted soil. In comparison to 3SBE, another 6–8 % of applied radioactivity was extracted by PLE after 100 d of incubation for all soils.

In the following, the radioactivity remaining in the soil after PLE will be named as **PLE_NER**.

^{14}C-Fenoxycarb was rapidly mineralised in all three soils and the percentage of evolving $^{14}CO_2$ increased continuously. After 100 d of incubation between 48 % (Lufa 2.2), 43 % (Lufa 2.3) and 40 % (Lufa 2.4) of the originally applied radioactivity were mineralised to $^{14}CO_2$. However, $^{14}CO_2$ formed within the first 15 d of incubation, accounted for about two-thirds of the total quantity of $^{14}CO_2$ observed within 100 d of the experiment for all soils. The quantity of radioactivity extractable using 3SBE decreased rapidly as a function of incubation time. After 1 day of incubation 34–65 % of applied radioactivity were extractable (EF), whereas after 15 d only 9 - 13 % and after the full incubation time of 100 d only 5–8 % of the applied radioactivity remained extractable. As the EF decreased, NER was rapidly formed after application of ^{14}C-fenoxycarb and did not change significantly between day 4 and day 100. The quantities of NER related to fenoxycarb were similar in all three test soils resulting in 45 % NER in Lufa 2.2, 51 % NER in Lufa 2.3 and 55 % in Lufa 2.4. The parent compound fenoxycarb as well as its transformation product (TP) hydroxy-fenoxycarb could be identified and quantified. In all soils ^{14}C-Fenoxycarb was rapidly transformed with DT_{50}-values < 3 d and DT_{90}-values < 11 d, as only 6–8 % of the radioactivity initially applied was present as fenoxycarb after 11 d of incubation. After batch extraction (3SBE), only minor fractions of 2–3 % of applied radioactivity were additionally extractable by PLE after 100 d (Table 8).

For ^{14}C-acetaminophen the percentage of evolving $^{14}CO_2$ increased continuously. After the entire incubation time of 35 d 14 % (Lufa 2.2), 18 % (Lufa 2.3) and 11 % (Lufa 2.4) of the originally applied radioactivity were mineralised to $^{14}CO_2$. Acetaminophen was quickly bound to the soil forming NER. Already a few hours after spiking, only 3–5 % (see table A68 – A70) of the applied radioactivity was extractable using 3SBE and only 2 % of the radioactivity was extractable after 35 d, resulting in 87–95 % NER. A subsequent PLE provided 3–4 % of the applied radioactivity while 84–91 % remained in the soil after PLE.

Variability of NER

As stated earlier, NER are operationally defined by the extraction procedure used. Up to now there is no exact and general standard for the extraction procedure, defining where the extractable fraction ends and the non-extractable residue begins. When talking about NER the respective extraction procedure has always to be mentioned.

In order to illustrate the relevance of the extraction procedure for the variability of the NER determination, our results for triclosan, fenoxycarb and acetaminophen were used.

The 3SBE combines 3 extraction procedures of increasing intensity to one sequential method. Additionally, PLE was applied as a fourth extraction step. Since the radioactivity extractable by each step was determined individually, the corresponding proportions of NER can be compared for all 4 extraction steps, representing the entire range of extraction efficiencies as obtained within the testing of organic chemicals in soils matrices. This means, depending on the extraction procedure used, the NER fraction related to triclosan in Lufa 2.2 after 100 d of incubation, varied between 96 % and 28 %. A similar behaviour was found for triclosan in the other two soils. For fenoxycarb, the variability of NER depending on the extraction procedure was smaller, with values between 52 % and 41 % NER in Lufa 2.2, as the extractability of fenoxycarb was generally lower.

It was highlighted that the percentage of NER reported for a compound can show a strong variability with the extraction procedure used, with possible consequences for the environmental risk assessment of this compound. Degradation/disappearance times can be directly affected by the extraction procedure, as more intense extraction procedures may release a higher proportion of a test compound and its TP, leading to increased values for the degradation time, which may be of consequence for the persistence assessment of that compound.

Frequently, the fraction of NER can easily be directed to a certain extent by the experimentalist by applying an extraction procedure “suitable” for purpose and outcome of the study, possibly overestimating the proportion of the NER fraction. Therefore, it would be valuable to perform a standardised, strong and exhaustive extraction procedure in order to improve the comparability of the data and prevent an overestimation of the NER fraction.

Consequences for the extraction Procedure

As an alternative, the optimised PLE procedure only (= direct PLE) was performed and the results were compared with those obtained from sequential batch extraction and consecutive PLE (3SBE&PLE).

It showed, that direct PLE and 3SBE&PLE provided the same results for the extractable and the non-extractable fractions (PLE_NER) of soil incubated with triclosan and fenoxycarb, considering the standard deviations of both procedures.

The application of PLE with a ternary extraction agent (methanol/acetone/water, 50/25/25, v/v/v) provided a widely exhaustive extraction, excellent extraction efficiencies for a wide range of organic chemicals and a low variability of the analytical data, using a single standardised extraction step.

Consequently, the direct PLE (PLE alone) offers a good alternative to the sequential extraction procedure.

Characterisation of PLE_NER

In additional experiments, the PLE_NER was characterised using silylation/EDTA extraction covering physically entrapped residues in the soil and those covalently bound to the soil matrix. Additionally, an HCl-treatment was performed addressing biogenic residues.

It was found, that the percentage of radioactivity released by EDTA extraction was higher as compared to silylation. The most noticeable difference was found for acetaminophen for which significantly more radioactivity was mobilised by EDTA extraction than by silylation. While EDTA extraction generally allows for the analysis of parent compounds and TPs using radiotracer methodology, for silylation this is much more difficult due to chemical alterations of the analytes and the matrix.

Residues from xenobiotics, e.g. pesticides, pharmaceuticals, industrial chemicals or biocides, which are transformed by the soil fauna and –flora into biomolecules and are incorporated into their biomass (e.g. proteins, fatty acids, nucleic acids, sugars and amino sugars), are called biogenic residues including biogenic NER. Since the compounds are transformed into biomolecules, no possible environmental risk is anticipated. A possible risk for the environment from extractable and non-extractable residues is expectable only for the parent compound and its transformation products. A standard approach for the determination of biogenic NER (e.g. proteins) in incubated and pre-extracted soil is acidic hydrolysis using hydrochloric acid, followed by a detailed analysis of the hydrolysate (e.g. for amino acids). In this study only the HCl-treatment was conducted, in order to verify whether the acidic hydrolysis released significant amounts of radioactivity, indicating the possible presence of biogenic NER.

For triclosan proportions of 4–23 % of applied radioactivity were released from PLE_NER within this step. In case of fenoxycarb proportions of 17–34 % and for acetaminophen 22–36 % of applied radioactivity were mobilised over all test soils and sampling times. The radioactivity, which was mobilised by means of the acidic hydrolysis is probably released due to different mechanisms. Beside the hydrolysis of any proteins in the soil, also other residues may be released. Especially for fenoxycarb and acetaminophen, being rapidly transformed, significantly mineralised and extensively forming NER, it may be assumed that transformation products of these compounds were significantly incorporated into microbial biomass.

Recommendations for a new NER-extraction scheme

From the results of the extraction experiments and the characterisation of NER it is recommended, to use a pressurised liquid extraction procedure (PLE) as a standard for the analysis of parent compounds and TPs in testing the fate of organic chemicals for regulatory purposes. The quite universal extraction procedure applies a ternary extraction agent consisting of methanol, acetone and water (50/25/25, v/v/v) and offers excellent extraction efficiencies for a broad range of compounds, a low variability of the results and high comparability in the analysis of soils.

The extract obtained by PLE can be assumed to contain all extractable compounds including the weakly sorbed and strongly sorbed fraction. The non-extractable residues in the soil after PLE, were named PLE_NER, in order to avoid confusion with NER obtained otherwise and contain residues physically entrapped in the soil matrix and covalently bound to soil particles and soil organic matter including biogenic NER. The physically entrapped NER are considered to be reversibly bound (Schaeffer et al., 2018) and can be characterised and quantified further by using procedures such as EDTA-extraction/silylation and amino acid extraction for an extensive NER-analysis.

Further research is necessary for a better understanding of processes leading to a release of sequestered residues during PLE, EDTA extraction and silylation and for further developments and standardisation in the determination and characterisation of biogenic NER.

Zusammenfassung

Große Mengen organischer Substanzen anthropogenen Ursprungs werden über unterschiedliche Expositionspfade in die Umwelt eingetragen. Pflanzenschutzmittel, wie Pestizide und Herbizide werden durch die Landwirtschaft direkt auf Böden appliziert. Dahingegen gelangen Biozide, Wasch- und Reinigungsmittel, Human- und Tierarzneimittel sowie Körperpflegeprodukte oft erst nach ihrer Anwendung über das Abwasser, Faulschlamm oder Gülleaufbringung in die Umwelt.

Während die Menge einer Substanz, die ursprünglich in ein Umweltkompartiment eingetragen wurde, meist mit der Zeit abnimmt, verschwinden dessen Rückstände jedoch nur selten vollständig. Viele Chemikalien werden im Boden und im Sediment immobilisiert und liegen dort dann als nicht extrahierbare Rückstände (NER) vor. Es wird angenommen, dass von den 4 Millionen Tonnen an Pflanzenschutzmitteln, die jährlich weltweit in der Landwirtschaft eingesetzt werden, etwa ein Drittel im Boden als NER zurückbleibt (Barriuso et al., 2008; Food and Agriculture Organization of the United Nations (FAO), 2019). Der Anteil einer Substanz, der als nicht-extrahierbar im Boden zurückbleibt, wird maßgeblich vom verwendeten Extraktionsverfahren bestimmt und wird von den experimentellen Parametern, wie den Inkubationsbedingungen, der Matrix und den jeweiligen Substanzeigenschaften beeinflusst (Barriuso et al., 2008; Gevao et al., 2003; Loos et al., 2012; Mordaunt et al., 2005; Northcott and Jones, 2000b).

Obwohl bekannt ist, dass die nicht extrahierbaren Rückstände anthropogen eingebrachter Substanzen durch ihre großen Mengen eine hohe Umweltrelevanz besitzen, gibt es in Deutschland und der EU kein einheitliches Verfahren, wie nichtextrahierbare Rückstände von Substanzen zu ermitteln und zu bewerten sind. Dies ist insbesondere von Relevanz für Chemikalien, die verschiedenen rechtlichen Regelungen unterliegen, wie z.B. für Industriechemikalien, Pflanzenschutzmittel, Human- und Tier-Arzneimittel und Biozide.

Experimenteller Ansatz

Das Ziel dieses Projektes war die Überprüfung des sequentiellen Extraktionsschemas zur Charakterisierung von nicht extrahierbaren Rückständen nach Eschenbach und Oing (Eschenbach and Oing, 2013b). Daneben sollte ein einheitlicher Ansatz zur Bestimmung von nicht extrahierbaren Rückständen im Rahmen der Umweltrisikobewertung von Chemikalien entwickelt werden.

Dazu wurde ein umfangreicher experimenteller Vergleich von verschiedenen Extraktionsverfahren und –methoden durchgeführt. Weiterhin wurden nicht-extrahierbare Rückstände von drei ^{14}C-markierten Substanzen (Triclosan, Fenoxycarb und Acetaminophen) analysiert, welche in Bodentransformationsstudien nach OECD Richtlinie TG 307 gebildet wurden. In allen Experimenten wurden die Böden Lufa 2.2, Lufa 2.3 und Lufa 2.4 verwendet, die hinsichtlich ihrer Textur einen breiten Bereich abdecken.

Vergleich der Extraktionstechniken

Zum Vergleich der verschiedenen Extraktionsverfahren und -methoden wurden 42 organischen Substanzen unterschiedlicher physikochemischer Eigenschaften und Anwendungsbereiche auf die drei genannten Böden dotiert, 9 Tage inkubiert, extrahiert und mittels LC-MS/MS analysiert.

Die Studienergebnisse zeigten, dass die beschleunigte Lösemittelextraktion (PLE/ASE) bei 100°C und 100 bar, mit einem ternären Extraktionsmittel bestehend aus Methanol, Aceton und Wasser (50/25/25, v/v/v) die höchsten Extraktionseffizienzen aller angewendeten Techniken aufwies, und dabei der geringsten Variabilität unterlag. Durch dieses Verfahren wurden, weitestgehend unabhängig von Substanz und Bodentyp, annähernd quantitative Extraktionsergebnisse erhalten. Folglich könnte dieses Extraktionsverfahren für die Analyse vieler organischer Substanzen im Boden, im Rahmen der Stoffregistrierung nützlich sein.

Für einzelne Substanzen kann nicht ausgeschlossen werden, dass deren Extraktionseffizienzen bedingt durch ihre jeweiligen Eigenschaften zu niedrig sind (z.B. Thermolabilität). Daher ist die Anwendbarkeit jeglicher analytischen Methodik für jede untersuchte Substanz (einschließlich deren TP´s) und für jede Matrix sicherzustellen. Das hier genannte PLE Verfahren kann dabei zumindest als guter Ausgangspunkt für die Methodenoptimierung dienen.

Bodentransformationsstudien und NER Charakterisierung

Anschließend wurden Bodentransformationsstudien mit den Radiotracern ^{14}C-Triclosan, ^{14}C-Fenoxycarb und ^{14}C-Acetaminophen entsprechend der OECD Richtlinie 307 durchgeführt. Dabei wurden die drei oben genannten Standardböden eingesetzt, um nicht extrahierbaren Rückstände für eine anschließende Charakterisierung zu generieren.

Die Stoffe Triclosan, Fenoxycarb und Acetaminophen wurden gewählt, da bei ihnen bereits eine umfangreiche Bildung nicht extrahierbarer Rückstände beobachtet wurde und sie in unterschiedlichen Anwendungsgebieten zum Einsatz kommen.

Neben der Charakterisierung des Verbleibs und des Verhaltens von Triclosan, Fenoxycarb und Acetaminophen während der Inkubation über 100 bzw. 35 Tage wurden die gebildeten NER mittels sequentieller Schüttelextraktion, PLE, Silylierung, EDTA- und HCl-Extraktion charakterisiert.

Während aller Transformationsstudien wurde die eingesetzte Radioaktivität quantitativ wiedergefunden. Volatile Bestandteile, die in Paraffinfallen aufgefangen wurden, waren für alle drei Substanzen mit < 1 % der applizierten Aktivität vernachlässigbar.

Vom applizierten ^{14}C-Triclosan wurden innerhalb der Versuchsdauer von 100 Tagen zwischen 11 % und 27 % zu $^{14}CO_2$ mineralisiert. Eine dreistufige Schüttelextraktion (3SBE) mit einer gestaffelten Extraktion mit 0,01 M $CaCl_2$, Methanol/Wasser (50:50, v/v) und Methanol/Aceton (50:50, v/v) wurde zur quantitativen Bestimmung der extrahierbaren Anteile (EF) bzw. der nicht extrahierbaren Rückstände (NER) im inkubierten Boden verwendet. Der NER-Anteil nahm während der Inkubation kontinuierlich zu, während der extrahierbare Anteil (EF) entsprechend in allen Böden abnahm. Im Boden Lufa 2.2 nahm der extrahierbare Anteil nach 7 Tagen auf 84 % und nach 100 Tagen auf 64 % ab, während der NER-Anteil in der gleichen Zeit von 16 % auf 34 % der eingesetzten Gesamtaktivität zunahm. Im Vergleich dazu wurde in Lufa 2.3 eine signifikant höhere Menge an NER (56 %) gebildet. Durch die chemische Analyse mittels Radio-HPLC konnte gezeigt werden, dass Triclosan in das Transformationsprodukt (TP) Methyl-Triclosan (Me-TCS) umgewandelt wurde. Die berechneten Halbwertszeiten (DT_{50}) von Triclosan lagen zwischen 2,3 und 56 Tagen und die DT_{90}-Werte zwischen 19 und 185 Tagen.

Me-TCS wurde in Lufa 2.2 und 2.3 kontinuierlich und in ähnlichem Umfang gebildet und lag nach 100 Tagen zu 22 % bzw. 16 % vor. Dahingegen wurde für Me-TCS in Lufa 2.4 nach 34 Tagen ein Maximum bei 60 % beobachtet, wonach der Anteil von Me-TCS an Tag 100 bis auf 34 % abfiel. Durch die sehr starke Sorption von TCS und Me-TCS an die Bodenmatrix hängen die ermittelten Halbwertszeiten stark von der gewählten Extraktionsmethode ab. Mittels PLE wurden im Vergleich zur 3SBE zusätzliche 6-8 % an applizierter Radioaktivität aus den über 100 Tagen inkubierten Böden extrahiert.

Im Weiteren wird die nach PLE im Boden verbleibende Radioaktivität als **PLE_NER** bezeichnet.

Fenoxycarb wurde in allen 3 Böden schnell und kontinuierlich zu $^{14}CO_2$ mineralisiert. Nach einer Inkubationszeit von 100 Tagen waren zwischen 48 % (Lufa 2.2), 43 % (Lufa 2.3) und 40 % (Lufa 2.4) der applizierten Gesamtaktivität zu $^{14}CO_2$ mineralisiert. Bereits innerhalb der ersten 15 Tage kam es zur Bildung von zwei Drittel des insgesamt während der gesamten Inkubationszeit von 100 Tagen gebildeten $^{14}CO_2$. Der durch 3SBE extrahierbare Anteil nahm in Abhängigkeit von der Inkubationszeit schnell ab. Waren nach einem Tag noch 34 % - 65 % der Gesamtaktivität extrahierbar, nahm dies nach 15 Tagen auf 9-13 % ab und nach der gesamten Inkubationszeit von 100 Tagen waren nur noch 5 - 8 % der Gesamtaktivität extrahierbar. Während der extrahierbare Anteil abnahm, nahm der nicht

extrahierbare Anteil nach der Applikation von ^{14}C-Fenoxycarb schnell zu und unterlag zwischen Tag 4 und Tag 100 nur einer geringen Varianz. In allen drei Böden war der Anteil von aus ^{14}C-Fenoxycarb gebildetem NER mit Anteilen von 45% (Lufa 2.2), 51 % (Lufa 2.3) und 55 % (Lufa 2.4) vergleichbar. Die Ausgangssubstanz Fenoxycarb und ihr Transformationsprodukt (TP) Hydroxy-Fenoxycarb konnten mittels Radio-HPLC identifiziert und quantifiziert werden. In allen Böden wurde Fenoxycarb schnell, mit DT_{50}-Werten von < 3 d und DT_{90}-Werten von < 11 d, transformiert. Nur 6 - 8 % der initial applizierten Radioaktivität waren nach 11 Tagen Inkubation noch als Fenoxycarb vorhanden. Aus den über 100 Tagen inkubierten Böden konnten nach der 3SBE durch PLE nur ein sehr geringer zusätzlicher Anteil von 2 - 3 % der Gesamtaktivität extrahiert werden.

Bei ^{14}C-Acetaminophen nahm der Anteil des gebildeten $^{14}CO_2$ ebenfalls kontinuierlich zu. Nach der Inkubationszeit von 35 Tagen waren 14 % (Lufa 2.2), 18 % (Lufa 2.3) und 11% (Lufa 2.4) der initial applizierten Menge zu $^{14}CO_2$ mineralisiert. ^{14}C-Acetaminophen wurde schnell als NER in den Böden festgelegt. Schon wenige Stunden nach der Applikation waren nur noch 3 – 5 % der Gesamtaktivität mittels 3SBE extrahierbar. Nach 35 Tagen konnten nur noch 2 % der eingesetzten Radioaktivität extrahiert werden. Daraus resultierte die Bildung von 88–95 % NER. Eine anschließende PLE konnte weitere 3–4 % der Radioaktivität extrahieren, während 84–91 % der Gesamtaktivität nach der PLE als PLE_NER im Boden zurückblieben.

Variabilität der NER

Wie zuvor erwähnt, sind NER operativ durch die gewählte Extraktionsmethode definiert. Es gibt keine standardisierte Extraktionsmethode, welche den Umfang der extrahierbaren Fraktion und der nicht-extrahierbaren Fraktion definiert. Bei einer Angabe von NER-Daten, muss die angewendete Extraktionsmethode daher immer mit angegeben werden.

Die Abhängigkeit des resultierenden NER-Anteils vom gewählten Extraktionsverfahren konnte aus den Studiendaten von Triclosan, Fenoxycarb und Acetaminophen dargestellt werden.

Die dreistufige Batchextraktion vereint drei Extraktionen mit ansteigender Intensität zu einer sequenziellen Methode. Zusätzlich wurde die PLE als vierter Extraktionsschritt verwendet. Da die extrahierte Radioaktivität für jeden Extraktionsschritt einzeln bestimmt wurde, konnten die daraus resultierenden Anteile des NER für alle 4 Schritte verglichen werden. Diese vier Extraktionsschritte repräsentieren praktisch die komplette Bandbreite an Extraktionseffizienzen, die Extraktionsverfahren während der Testung von organischen Chemikalien im Boden und Sediment, zeigen können.

Die Variationsbreite des hier ermittelten NER Anteils lag, abhängig vom gewählten Extraktionsverfahren für Triclosan in Lufa 2.2 nach 100 Tagen, zwischen 96 % und 28 %. Ein ähnliches Verhalten wurde auch für Triclosan in den anderen beiden Böden festgestellt. Für Fenoxycarb wurde eine geringere Variabilität des NER Anteils, in Abhängigkeit von der verwendeten Extraktionsmethode, mit Werten zwischen 52 % und 41 % festgestellt, was daraus resultierte, dass Fenoxycarb generell eine geringere Extrahierbarkeit zeigte.

Die Untersuchungsdaten verdeutlichen, dass der Umfang an NER, der aus einer Substanz gebildet wird, eine starke Variabilität durch die verschiedenen eingesetzten Extraktionsverfahren aufweisen kann. Diese Variabilität kann einen Einfluss auf die Umweltrisikobewertung der Substanz haben, da auch Abbau- und Dissipations-Halbwertszeiten durch die gewählten Extraktionsverfahren direkt beeinflusst werden können. So können intensivere Extraktionsverfahren einen höheren Anteil der Testsubstanz bzw. von dessen Transformationsprodukten (TP) herauslösen, was größere Halbwertszeiten bedingen und sich so auf die ermittelte Persistenz der Substanz auswirken kann.

Der Anteil des NER kann, in gewissem Umfang, durch das eingesetzte Extraktionsverfahren beeinflusst werden, so dass er dem „gewünschten“ Zweck und Ergebnis der Studie dient, was ggf. mit einer „Überbestimmung“ des NER einhergeht. Um dies zu vermeiden, sollte ein starkes und erschöpfendes

Extraktionsverfahren angewendet werden. Dadurch könnte eine bessere Vergleichbarkeit der Daten geschaffen und eine Überbestimmung des NER weitgehend vermieden werden.

Konsequenzen für das Extraktionsverfahren

Das kombinierte Verfahren (3SBE&PLE) wurde mit der PLE-Methode alleine (direkte PLE) verglichen. Dabei zeigte sich, dass die Ergebnisse für den extrahierbaren und nicht extrahierbaren Anteil von Böden inkubiert mit Triclosan bzw. Fenoxycarb aus dem kombinierten Verfahren (3SBE&PLE) mit denen der einstufigen PLE vergleichbar sind.

Durch die hier vorgestellten Ergebnisse konnte gezeigt werden, dass die angewendete PLE-Methode mit einem ternären Lösemittelgemisch (Methanol/Aceton/Wasser, 50/25/25, v/v/v) eine weitestgehend erschöpfende Extraktion, mit einer hervorragenden Extraktionseffizienz für ein breites Spektrum organischer Substanzen liefert und dabei nur einer vergleichsweise geringen Variabilität der analytischen Daten unterliegt. Dementsprechend zeigen die mit dieser einstufigen Extraktion gewonnenen Daten eine hohe Vergleichbarkeit.

Folglich stellt die direkte PLE (PLE alleine) eine generelle Alternative zur sequentiellen Extraktion dar.

Charakterisierung der PLE_NER

In ergänzenden Experimenten wurde der PLE-NER mittels Silylierung/EDTA-Extraktion charakterisiert, um physikalisch eingeschlossene und kovalent gebundene Rückstände zu bestimmen. Weiterhin wurde eine Behandlung des PLE_NER mit HCl zur Adressierung der biogen gebundenen NER durchgeführt.

Es zeigte sich, dass der Prozentsatz an Radioaktivität, der durch die EDTA-Extraktion freigesetzt wurde, im Vergleich zur Silylierung meist etwas höher war. Der auffälligste Unterschied wurde hierbei für die Substanz Acetaminophen beobachtet.

Während im Extrakt der EDTA Extraktion eine Bestimmung von Ausgangssubstanzen und Transformationsprodukten mittels der Radiotracermethodik möglich ist, ist dies bei der Silylierung deutlich erschwert, da die Analyten und die Matrix hier durch die Silylierung chemisch verändert werden.

Rückstände von Xenobiotika wie Pestiziden, Arzneimitteln, Industriechemikalien oder Bioziden, die durch Mikrobiom und Pflanzen in Biomoleküle umgewandelt und in die Biomasse eingebaut werden (z.B. Proteine, Fettsäuren, Nukleinsäuren, Zucker und Aminozucker) nennt man biogene Rückstände (inkl. biogene NER).

Ein mögliches Umweltrisiko durch extrahierbare und nicht extrahierbare Rückstände ist nur für die Ausgangssubstanz und ihre Transformationsprodukte zu erwarten, für biogene NER jedoch nicht.

Zur Bestimmung von biogenen NER (z. B. Proteinen), kann Boden einer sauren Hydrolyse unter Verwendung von Salzsäure unterzogen werden, gefolgt von einer Analyse des Hydrolysats (z. B. auf Aminosäuren). In dieser Studie wurde lediglich die saure Hydrolyse durchgeführt, um zu überprüfen, ob durch diese signifikante Mengen an Radioaktivität freigesetzt werden, was auf die mögliche Anwesenheit von biogenen NER hinweisen könnte.

Durch die saure Hydrolyse wurden für Triclosan Anteile zwischen 4 – 23 % der applizierten Radioaktivität aus dem PLE_NER freigesetzt. Bei Fenoxycarb wurden Anteile von 17 – 34 % und bei Acetaminophen 22 – 36 % der applizierten Radioaktivität über alle Testböden und Probenahmezeitpunkte mobilisiert. Die Radioaktivität, die durch die saure Hydrolyse mobilisiert wurde, wird wahrscheinlich aufgrund verschiedener Mechanismen freigesetzt. Neben der Hydrolyse jeglicher Proteine im Boden können auch andere Rückstände freigesetzt werden. Insbesondere für Fenoxycarb und Acetaminophen, die schnell transformiert wurden, einer signifikanten

Mineralisierung unterlagen und umfangreich NER bildeten, kann angenommen werden, dass Abbauprodukte dieser Verbindungen signifikant in mikrobielle Biomasse eingebaut wurden.

Empfehlungen für ein neues NER-Extraktionsschema

Ausgehend von den Ergebnissen der Extraktionsstudien und der Charakterisierung der NER wird vorgeschlagen, die PLE als Standardverfahren für die Analyse von Ausgangssubstanzen und deren Transformationsprodukten zur Bestimmung des Verbleibs und des Verhaltens organischer Chemikalie in der Umwelt zu verwenden. Das vorgeschlagene Extraktionsverfahren, das ein ternäres Extraktionsmittel aus Methanol, Aceton und Wasser (50/25/25, v/v/v) verwendet, ist weitgehend universell verwendbar, da es ausgezeichnete Extraktionseffizienzen für eine breite Palette von Verbindungen und Böden liefert, nur einer relativ geringen Variabilität der Ergebnisse unterliegt und damit eine hohe Vergleichbarkeit in der Analyse von Böden und Sedimenten bietet.

Der aus der Extraktion mit dieser PLE Methode erhaltene Rückstand wird als PLE_NER benannt, um Verwechslungen mit NER aus sonstigen Extraktionsverfahren zu vermeiden. Es ist davon auszugehen, dass der Extrakt, der nach der beschriebenen PLE vorliegt, alle extrahierbaren Verbindungen, einschließlich der schwach und stark sorbierten Fraktion enthält. PLE_NER enthält physikalisch eingeschlossene NER und NER, die kovalent an Bodenpartikeln oder organische Bodensubstanz gebundenen sind, sowie biogene NER. Der physikalisch eingeschlossene Anteil der NER gilt als reversibel gebunden (Schaeffer et al., 2018) und kann für eine weitergehende NER-Analyse unter Nutzung von Verfahren wie EDTA-Extraktion/Silylierung und Aminosäureextraktion charakterisiert bzw. quantifiziert werden.

Weiterer Forschungsbedarf ist bei einer Verbesserung des Verständnisses von Prozessen zu sehen, die zu einer Freisetzung von Stoffen aus stark sorbierten Rückständen und physikalischen Einschlüssen bei der PLE, EDTA-Extraktion und Silylierung führen. Weiterhin sollte die Entwicklung und Standardisierung von Techniken zur Bestimmung und Charakterisierung von BioNER vorangetrieben werden.

1 Introduction

A wide range of anthropogenic organic compounds is deliberately introduced into the environment. Agricultural products, e.g. pesticides and herbicides, are directly applied onto soils and detergents, pharmaceuticals and personal care products enter the environment mainly via wastewater, digested sludge or manure (Bloem et al., 2017; der Beek et al., 2016; Jardak et al., 2016; Kaczala and Blum, 2016; Kuppusamy et al., 2018; Prosser and Sibley, 2015; Schaider et al., 2017; Scott and Jones, 2000; Tran et al., 2018; Wohde et al., 2016; Zhang and Li, 2011).

Once in the environment, these organic compounds are subjected to several fate processes including transport processes, biotic and abiotic transformation, sorption and leaching. Soils and sediments play an important role in these fate processes, providing a wide variety of binding sites and are the major sinks for many of these compounds (Jablonowski et al., 2009; Kaestner, 2000; Northcott and Jones, 2000b).

While the amount of a compound in an environmental compartment is observed to decrease with time, it rarely disappears entirely. Organic chemicals are known to be immobilised or sequestered in contact with soil or sediment forming non-extractable residues (NER) (Kaestner et al., 2014; Northcott and Jones, 2000b).

It can be estimated that about one third of the 4 million tons of pesticides (active ingredients) which are annually applied worldwide remain in agricultural soils as NER (Barriuso et al., 2008; Food and Agriculture Organization of the United Nations (FAO), 2019), emphasizing the environmental relevance of NER.

Over the years non-extractable or bound residues have been defined by IUPAC in different ways (Calderbank, 1989; Mordaunt et al., 2005; Roberts, 1984). Führ *et al.* expanded the definition to include reference to the structure of the matrix (Führ et al., 1998): ‘‘Bound residues represent compounds in soil, plant or animal, which persist in the matrix in form of the parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the nature of the matrix. The nature of the bond can be clarified in part by matrix altering extraction methods and sophisticated analytical techniques. To date, for example, covalent, ionic and sorptive bonds as well as entrapments have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and bioavailability significantly.’’

Unfortunately, the definition provides no exact recommendation on the extraction methods to be used, avoiding “*a substantial change of the nature of the matrix”.* Even as far back as 1976, Kaufman stated that “the definition or interpretation of what a bound residue was, varied with each individual scientist and the extraction used” (Kaufman, 1976) which holds true more than forty years later.

The quantitative proportion of a compound which remains non-extractable is operationally defined by the extraction procedure employed. It is influenced by experimental/environmental parameters such as incubation conditions, matrices and compounds investigated (Barriuso et al., 2008; Gevao et al., 2003; Loos et al., 2012; Mordaunt et al., 2005; Northcott and Jones, 2000b).

NER are usually determined by radiotracer analysis, as by radiolabelling a complete mass balance can be determined including the formation of NER, mineralisation, transformation and distribution processes (Slater and Slater, 2002; Wang et al., 1975). In radiotracer analysis batch extraction is frequently used for the analysis of soils, although it provides only a comparably low extraction intensity.

Mechanistical aspects of the formation of non-extractable residues have been reviewed by several authors giving insight into the interactions between organic chemicals and soil (Calderbank, 1989; Gevao et al., 2000; Kaestner et al., 2014; Klaus et al., 1998).

In NER literature a variety of terms are used to describe fractions of NER in soil, e.g. strongly bound (Achtnich et al., 1999a; Calderbank, 1989; Dec and Bollag, 1997; Jablonowski et al., 2012; Kaestner et al., 2014; Klaus et al., 1998; Schaeffer et al., 2018; Stokes et al., 2005; Umeh et al., 2017), strongly sorbed (Bourdat-Deschamps et al., 2017; Kah and Brown, 2007; Reichenberg et al., 2006; Schaeffer et al., 2018; Wang et al., 2016), heavily sorbed (ECHA, June 2017; Eschenbach and Oing, 2013b), irreversibly sorbed (Ahmad et al., 2004; Alexander, 1995; ECETOC, 2013a, b; Suddaby et al., 2016; Umeh et al., 2017), slowly desorbable (ECETOC, 2013a, b; Kaestner et al., 2018), very slowly desorbable (ECETOC, 2013b), entrapped (Achtnich et al., 1999a; Alexander, 2000; Eschenbach et al., 1998; Jablonowski et al., 2012; Northcott and Jones, 2000b; Schaeffer et al., 2018; Schaeffer et al., 2015; Steinberg et al., 1987) and sequestered (Dec and Bollag, 1997; Forster et al., 2009; Hartlieb et al., 2003; Kaestner et al., 2014; Rosendahl et al., 2011; Schaeffer et al., 2018; Sittig et al., 2012; Suddaby et al., 2016).

However, these NER fractions show an overlap with other NER fractions and are not generally but mostly operationally defined which applies also for the corresponding total extractable fractions (Al-Rajab et al., 2009; Barriuso et al., 2008; Cabrera et al., 2012; ECETOC, 2013a; Northcott and Jones, 2001; Schantz, 2006; Umeh et al., 2018; Umeh et al., 2019; Waria et al., 2011). Due to that a complete separation of the various NER fractions is hardly possible.

As bioavailability decreases with aging, non-extractable residues may accumulate in the soil and thus be released later leading to adverse effects e.g. on plants and (soil)organisms. The formation of non-extractable residues from an organic compound is of relevance for its persistency (Loeffler et al., 2018).

Hence, knowledge on the non-extractable residues of organic compounds is crucial for the assessment of their environmental behaviour and risk, and is used for the legal regulation of these chemicals (Barraclough et al., 2005; Craven, 2000; Craven and Hoy, 2005).

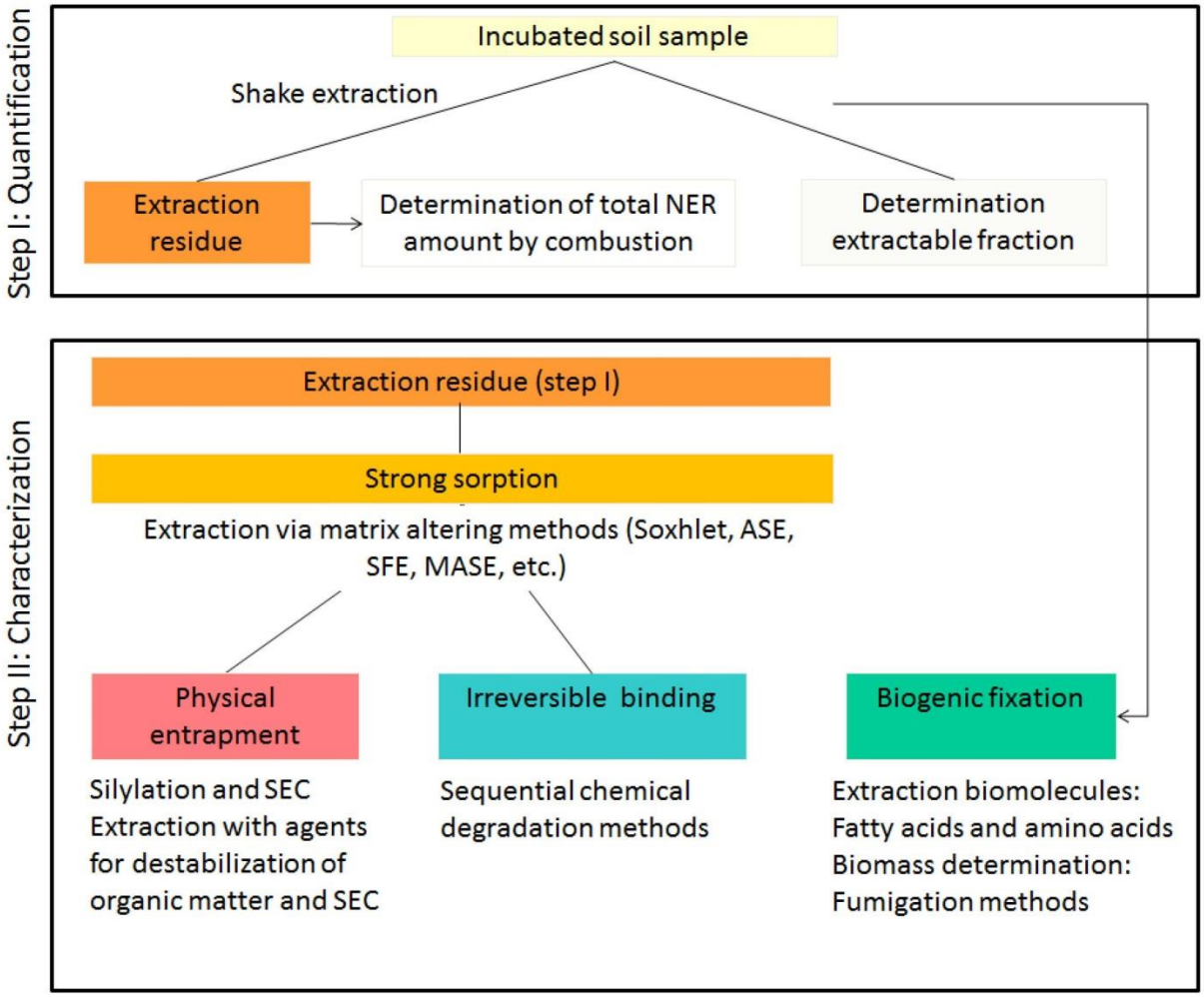
However, in German and European Union regulations there is no common agreement, on how to determine and how to assess data on the non-extractable residues (NER) especially when different legal regulations apply (e.g. for industrial chemicals (REACH), pesticides, biocides and pharmaceuticals) (Schaeffer et al., 2018; Wiemann, 2018; Wiemann et al., 2018).

It is therefore beneficial to develop a standardised procedure for the determination of NER, for unifying and simplifying their risk assessment and legal regulation (Craven and Hoy, 2005).

In this light, Barriuso et al. demanded that “A significant effort toward greater standardisation of experimental protocols is needed so that robust comparisons of data originating from different laboratories can be performed” (Barriuso et al., 2008).

Working on that issue, Eschenbach and Oing conducted a literature survey resulting in a sequential extraction scheme for the characterisation of non-extractable residues of organic compounds (Eschenbach and Oing, 2013b).

Figure 1: Sequential extraction scheme for the characterisation of non-extractable residues by Eschenbach and Oing (Eschenbach and Oing, 2013b)



Reference: Eschenbach and Oing (Eschenbach and Oing, 2013b)

In step I of this scheme, soil incubated with an organic chemical is subjected to batch extraction for the determination of total NER in the soil and the extractable fraction.

In step II, the NER formed during incubation of the organic compound is characterised by a sequential procedure. For that the extracted soil is extracted again using a harsh procedure such as soxhlet extraction, PLE, SFE, MASE allowing for a quantification of the proportion underlying a strong sorption to soil and/or soil organic matter. The twice extracted soil is estimated to contain substances which are (1) physically entrapped in the soil matrix and (2) covalently and thus irreversibly bound to the soil matrix. The entrapped proportion may be released by silylation (Dec et al., 1997b; Haider et al., 2000; Haider et al., 1992; Haider et al., 1993) and/or EDTAs extraction (Achtnich et al., 1999b; Eschenbach et al., 1998; Weiss et al., 2004) and needs to be analysed further. Sequential chemical degradation is used for a characterisation of those shares covalently bound to the soil or soil organic matter. Furthermore, compounds underlying biogenic fixation shall be analysed (Brock et al., 2017; Kaestner et al., 2014; Nowak et al., 2013; Nowak et al., 2011; Poßberg et al., 2016; Schaeffer et al., 2018; Trapp et al., 2017; Wang et al., 2016; Wang et al., 2017b; Wang et al., 2017c).

However, the applicability of this scheme needs to be experimentally confirmed.

1.1 Aim of this study

The aim of this project is i) the experimental verification of the extraction scheme for the determination of NER proposed by Eschenbach and Oing (Eschenbach and Oing, 2013b) and ii) to develop an overall extraction procedure.

Therefore, a widely universal extraction procedure was developed, being appropriate for a variety of different compounds and soils. This procedure was then used for the analysis of three ^{14}C-labelled test compounds within soil transformation experiments conducted according to OECD TG 307. After extraction, NER in the soils were characterised by silylation, EDTA extraction and HCl treatment.

2 Initial considerations

Prior to the various experiments, which had to be accomplished within this study, soils and ^{14}C-labelled test substances had to be selected.

2.1 Selection of soils

The selection of soils was based on evaluation criteria which might also affect the formation of non-extractable residues (NER):

- broad range of pH values of the soils
- broad range of soil textures
- broad range of C_{org} contents
- commercial availability and usage of the soils in standard tests

Based on these criteria, the following three soils were selected for all following experiments: Lufa 2.2, Lufa 2.3 and Lufa 2.4 (chapter A2.2). Their properties are summarised in Table 1.

Initial experiments with these soils resulted in low extractable fractions after the incubation period. The mean values for the extracted proportions of the fourteen spiked and evaluated substances were 57±30 %, 50±25 %, and 30±24 % (Table A4). These lower proportions being extracted indicate the formation of higher fractions of NER. The soils cover a wide range of different soil properties: the soil texture ranged from loamy sand (Lufa 2.2) to clayey loam (Lufa 2.4), the C_{org} content ranged from 0.67 (Lufa 2.3) to 1.99 (Lufa 2.4) and the pH value of the soils ranged from 5.4 (Lufa 2.3) to 7.4 (Lufa 2.4). Advantageous for the usage of these standard soils is their commercial availability.

Table 1: Physicochemical properties of the three soils used for the incubation experiments (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, 2016).

	Lufa 2.2	Lufa 2.3	Lufa 2.4
C_{org} (%)	1.61 ± 0.15	0.67 ± 0.03	1.99 ± 0.21
N (%)	0.17 ± 0.01	0.08 ± 0.01	0.22 ± 0.02
pH (-)	5.4 ± 0.2	5.9 ± 0.6	7.4 ± 0.1
CEC (meq/100g)	9.7 ± 0.4	7.6 ± 0.8	32.9 ± 4.5
Sand (%)	76.2 ± 0.4	59.6 ± 1.4	33.1 ± 2.2
Silt (%)	15.8 ± 3.1	33.6 ± 0.5	41.1 ± 1.2
Clay (%)	8.0 ± 1.7	6.8 ± 1.6	25.8 ± 1.8
Texture according to German DIN	loamy sand	silty sand	clayey loam
Texture according to USDA	sandy loam	sandy loam	loam

Reference: Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, *Analyses Data Sheet for Standard Soils according to GLP*. 2016

2.2 Substance selection

For the selection of three test substances the available information on a multitude of organic chemicals were evaluated regarding their field of application, usage quantity, chemical structure, physicochemical properties, persistence, mineralisation and their ability to form non-extractable residues (Table 2). Data for this selection process was 1) provided by UBA, 2) taken from literature and 3) resulted from incubation experiments performed at BfG (chapter A. 2.1).

Table 2: List of potential target substances to be used for the soil incubation experiments

Substance	CAS number	Main application
Acetaminophen	103-90-2	Analgesic
Amprolium	137-88-2	Coccidiostat
Benzyldimethyldodecylammonium*	139-07-1	Disinfectant
Carbendazim	10605-21-7	Fungicide
Climbazole	38083-17-9	Fungicide
Dimethomorph	110488-70-5	Fungicide
Ethinylestradiol	57-63-6	Estrogen
Fenoxycarb	72490-01-8	Insecticide
Fenpropimorph	67564-91-4	Fungicide
Florfenicol	76639-94-6	Antibiotic
Flumequine	42835-25-6	Antibiotic
Isoproturon	34123-59-6	Herbicide
Ketoconazole	65277-42-1	Fungicide
Mebendazole	31431-39-7	Antihelmintic
Mesosulfuron methyl	208465-21-8	Herbicide
Propiconazole	60207-90-1	Fungicide
Triclosan	3380-34-5	Disinfectant

* either bromide or chloride, CAS number is provided for the chloride

Reference: Federal Institute of Hydrology (2019)

2.2.1 Triclosan

As first test compound, the biocide triclosan (TCS) was applied (Figure 2). Triclosan is a widely used antibacterial and antifungal agent in personal care products such as soaps or toothpastes (Glaser, 2004; Halden and Paull, 2005; Miller et al., 2008).

During biological wastewater treatment, in soil and sediments TCS is transformed to the main transformation product (TP) methyl-triclosan, which is widely stable in the environment (Butler et al., 2012; Chen et al., 2011; Huang et al., 2014). Triclosan is a non-polar substance with a log K_{OW} of 4.8 and log K_{OC} of 4.6 (Wick et al., 2011). Therefore, it sorbs strongly to most solid matrices such as soil.

Up to 80 % NER were observed after 40 d in experiments with ^{14}C-labelled TCS in soils, while the mineralization rate was less than 15 % (Al-Rajab et al., 2009). During first experiments, the residual extractable fraction for this compound after 33 d of incubation was always <66 % and the dissipation

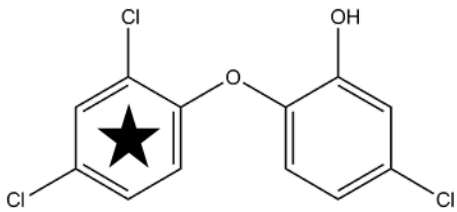
was potentially caused by a combined effect of a transformation of triclosan as well as the formation of NER (A2.4.17).

Both triclosan and its major TP methyl-triclosan are supposed to exhibit unspecific/non-ionic interactions with the soil matrix.

Crucial for the selection of the antibacterial agent triclosan was

- its tendency to form non-extractable residues,
- that it is a representative of the class of non-polar and non-ionic organic substances
- its persistence and
- its high sorption affinity.

Figure 2: Chemical structure of the biocide triclosan ^{14}C(U)-labelled in the 2,4-dichlorophenyl moiety [1]



Reference: Federal Institute of Hydrology (2019)

2.2.2 Fenoxycarb

Another test compound selected was the insecticide fenoxycarb (FEC) (Figure 3) which is widely applied as an insect growth regulator in fruit growing and vinery and is also used as ingredient in wood protection agents. The pK_a of fenoxycarb is 12.1 and the log K_{OW} and log K_{OC} provided in the EPI Suite are 4.3 and 3.3-3.7 (US EPA, 2012).

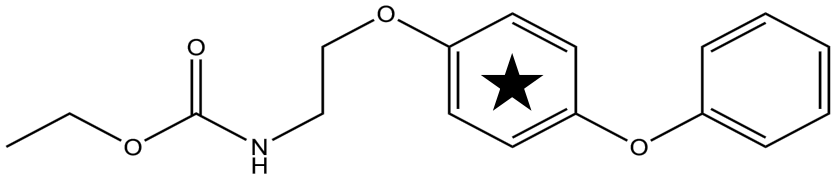
Scientific reports on the occurrence and fate of fenoxycarb in the aquatic and terrestric environment is relatively scarce (Sullivan, 2010). However, fenoxycarb possesses a high potential to generate NER in soils: data provided by UBA referred to a formation of 41–68 % NER after incubation in different soils over about 90 d and similar values were provided by EFSA (European Food Safety Authority (EFSA), 2010). The DT_{50} of fenoxycarb in these experiments ranged from 0.75–3 d with a mineralisation of 30 % after 90 d (European Food Safety Authority (EFSA), 2010). During initial experiments performed by BfG, the average extractable fraction of fenoxycarb using PLE was only 27 % while in soil Lufa 2.4 only 4 % were recovered after 33 d (cf. Chapter A2.4.8).

Crucial for the selection of the insecticide Fenoxycarb was

- its high tendency to form non-extractable residues,
- that it is a representative for organic substances that exhibit both specific bonding to the soil matrix via the nitrogen (e.g. covalent bond) as well as nonspecific interactions from the unpolar part of the molecule (e.g. van der Waals forces).

Figure 3: Chemical structure of the insecticide fenoxycarb 14C(U)-labelled in the bis-oxybenzene moiety [2]

[1&2] The asterisk marks the ^{14}C labelled phenyl ring.



Reference: Federal Institute of Hydrology (2019)

2.2.3 Acetaminophen

Finally, the analgesic drug acetaminophen (ACT, Figure 4) was selected, which is widely known as paracetamol. Acetaminophen is a very polar substance (log K_{OW} = 0.5, log K_{OC} = 1.3–1.5 (US EPA, 2012)) with a pK_a of 9.4 (Wan et al., 2003).

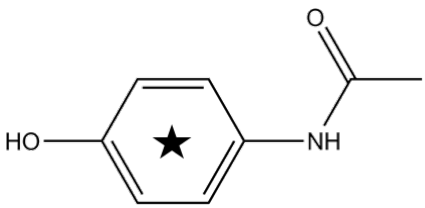
During incubations with soil and sediment a rapid transformation of acetaminophen was observed ($DT_{50,soil}$ < 1 d and $DT_{50,sediment}$ ~ 3 d) (Li et al., 2014; Loeffler et al., 2005b). The biologically controlled formation of NER of acetaminophen was observed in a study conducted by Li *et al*. (Li et al., 2014).

During initial experiments, acetaminophen was rapidly degraded and after 15 d less than 10 % of the initial concentration of acetaminophen was recovered in the six different soils applied.

Crucial for the selection of the pharmaceutical acetaminophen was

- its high tendency to form non-extractable residues,
- that it is a representative of substances that are well biodegraded and for which NER will potentially be formed mainly by biological processes and enhanced inclusion of small fractions of the molecule into microbial biomass.

Figure 4: Chemical structure of the analgesic drug acetaminophen $^{14}C(U)$-labelled in the phenyl-moiety [3]



Reference: Federal Institute of Hydrology (2019)

[2,3] The asterisk marks the ^{14}C-labelled phenyl ring.

3 Experimental comparison study of soil extraction methods

The selection of an extraction procedure is extremely important for the determination of the NER, as the intensity of the extraction procedure defines whether a soil bound fraction of an organic chemical is considered extractable or non-extractable.

To allow for a comparison of different extraction procedures a variety of organic compounds was spiked onto 3 different soils, incubated for 9 d, dried, ground, extracted and analysed by LC-MS/MS analysis.

3.1 Selection of compounds

The test compounds of this experiment were selected mainly with the aim to cover a broad range of polarity. For that, charged and non-charged compounds were chosen, covering a wide log K_{OW} range from -1.22 to 4.93. The compounds were also selected regarding their chemical functional groups. The selected compounds comprised primary and secondary alcohols, amines, amides as well as acids, carbamates and ethers. Finally, they cover different various fields of application such as pharmaceuticals, biocides, herbicides and industrial chemicals. The selected compounds are shown in Table 3.

Table 3: Compounds selected for the extraction study

Compound	Substance group	Internal standard	log K_{OW} value
Methoxymethyltriphenyl-phosphonium chloride	O / reagent in organic synthesis	Sitagliptin-d4	-1.22
Methyltriphenylphosphonium bromide	O / reagent in organic synthesis	Methyl-d3-triphenylphosphonium bromide	-1.18
Tetrapropylammonium	O / reagent in organic synthesis	Tetra-d28-propylammonium bromide	-0.30 (Vesta Intracon bv)
Denatonium	O / bitterant	Flecainid-d3	-0.04 (Pest Management Regulatory Agency, 2011)
Solatol	P / beta blocker	Metoprolol-d7	0.24 (Tetko et al., 2005)
Fluconazole	P / Anti mycotic	Fluconazole-d4	0.25 (US EPA, 2004)
Imidacloprid	B / insecticide	Imidacloprid-d4	0.57 (Tomlin and British Crop Protection, 2004)
Metamitron	B / herbicide	Metamitron-d5	0.83 (US EPA, 2012)
Sulfamethoxazole	P / antibiotic	Sulfamethoxazole-d4	0.89 (Hansch et al., 1995)
Primidone	P / anticonvulsant	Primidone-d5	0.91 (Hansch

Compound	Substance group	Internal standard	log K_{OW} value
			et al., 1995)
Amisulpride	P / antipsychotic	Amisulprid d5	1.1 (Sangster, 2013)
Sitaglipin	P / antidiabetic	Sitagliptin-d4	1.39 (EPA, 2012; US EPA, 2012)
Tetrabutylammonium	O / reagent in organic synthesis	Oxazepam-d5	1.60 (Roth, 2015)
Metoprolol	P / beta blocker	Metoprolol-d7	1.88 (Hansch et al., 1995)
Carbanilide	O / cytokine	DEET-d7	2.02 (Hansch et al., 1995)
DEET	O / insect repellent	DEET-d7	2.02 (Hansch et al., 1995)
Metazachlor	B / herbicide	Metazachlor-d6	2.13 (BASF, 2012)
Oxazepam	P / anxiolytic	Oxazepam-d5	2.24 (Hansch et al., 1995)
Carbamazepine	P / antiepileptic	Carbamazepine-15N13C	2.25 (Jones et al., 2002)
Aliskiren	P / renin inhibitor	Aliskiren-d6	2.45 (O'Neil and Royal Society of, 2013)
Clarithromycin	P /antibiotic	Clarithromycin-N-methyl-d3	2.6 (Hanisch, 2002)
Diuron	B / herbicide	Diuron-d6	2.68 (Hansch et al., 1995)
Mebendazole	P / antihelmintic	Mebendazole-d3	2.83
Isoproturon	B / herbicide	Isoproturon-d6	2.87 (Hansch et al., 1995)
Diazepam	P / anxiolytic	Diazepam-d5	2.99 (Hansch et al., 1995)
Naproxen	P / anti-inflammatory drug	Naproxen-d3	3.0 (Hanisch, 2002)
Irgarol	B / fungicide, algaecide	Irgarol-d9	3.1 (BASF, 2006)
Metolachlor	B / herbicide	Metolachlor-d6	3.13 (Hansch et al., 1995)
Diphenhydramin	P / antihistamine	Citalopram-d4	3.27 (Hansch et al., 1995)
Climbazole	B / antimycotic	Climbazol-d4	3.33 (Richter et al., 2013)

Compound	Substance group	Internal standard	log K_{OW} value
Terbuthylazine	B / herbicide	Terbutylazine-d5	3.40 (MacBean, 2004-2005)
Epoxiconazol	B / fungicide	Epoxiconazol-d4	3.58 (US EPA, 2006)
Tebuconazole	B / fungicide	Tebuconazole-d6	3.70 (Tomlin and British Crop Protection, 2004)
Propiconazole	B / fungicide	Propiconazole-d5	3.72 (Tomlin and British Crop Protection, 2004)
Terbutryn	B / herbicide	Terbutryn-d5	3.74 (Hansch et al., 1995)
Citalopram	P / antidepressant	Citalopram-d4	3.74 (Meylan and Howard, 1995)
Clopidogrel	P / antiplatelet agent	Clopidogrel-d4	3.80 (US EPA, 2004)
Azithromycin	P / antibiotic	Azythromycin-d3	4.02 (McFarland et al., 1997)
Fenoxycarb	B / insecticide	Epoxiconazol-d4	4.30 (Hansch et al., 1995)
Diclofenac	P / anti-inflammatory drug	Diclofenac-d4	4.51 (Hazardous Substances Data Bank (HSDB))
Triclocarban	B / antibacterial agent	Triclocarban-d4	4.90 (US EPA, 2012)
Fenpropimorph	B / fungicide	Oxazepam-d5	4.93 (Chamberlain et al., 1996)

P= Pharmaceutical, CP = chemical precursor, B = biocide, O = other,

Reference: Federal Institute of Hydrology (2019)

3.2 Experimental

3.2.1 Chemicals

The chemicals applied are given in table A5 of the annex.

3.2.2 Preparation and incubation of the soil samples

The same test soils (Lufa 2.2, Lufa 2.3 and Lufa 2.4) with the identical soil moistures were used in this study as in the OECD 307 study (chapter 4). A set of 42 compounds was chosen for this experiment (Table 3) being on the one hand a representative mixture of chemicals, biocides and pharmaceuticals and on the other hand covering several chemical properties such as log K_{OW} values and functional groups. The soil was spiked at a level of 20 ng/g dry mass for all compounds. The samples were incubated aerobically at room temperature for 9 d allowing sorption to soil and transformation. Before extraction the soil samples were lyophilised and ground ensuring the homogeneity of the samples. Samples of 1 g dry ground soil were subjected to the several extraction procedures described below. The extracts obtained were filled up to 50 mL (see chapter 3.2.3.1) and subjected to LC-MS/MS analysis after mixing with $^{2}H/^{13}C$-labelled compounds as internal standards (Table A3, Annex).

After determining the extracted concentrations of each compound in soil, a normalization of the results was necessary resulting in a data set independent of degradation of each compound and soil type.

3.2.3 Extraction procedures

The following extraction procedures were accomplished in triplicate for each soil:

3.2.3.1 Pressurised liquid solvent extraction:

A quantity of 1 g of dry soil was placed in an extraction cell (10 mL) and filled up with sea sand. Three consecutive extraction cycles, each 10 min, were performed using a Dionex, ASE 350 at a temperature of 100 °C and a pressure of 100 bar. The rinse volume was set to 60 %.

Following extracting agents were used with the same extraction parameters:

a) Single solvent extraction using isohexane. The combined extracts were filled up to 50 mL with isohexane. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution and evaporated to dryness and re-dissolved in methanol/water (50/50 (v/v)).

b) Single solvent extraction using ethyl acetate. The combined extracts were filled up to 50 mL with ethyl acetate. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution and evaporated to dryness and re-dissolved in methanol/water (50/50 (v/v)).

c) Single solvent extraction using acetone. The combined extracts were filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

d) Single solvent extraction using methanol. The combined extracts were filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

e) A binary solvent mixture composed from methanol/water (50/50 (v/v)). The combined extracts were filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

f) An acidified solvent mixture consisting of methanol/water/formic acid (50/50/1 (v/v/v)). The combined extracts were filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

g) A ternary solvent mixture consisting of methanol/acetone/water (50/25/25 (v/v/v)). The combined extracts were filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

3.2.3.2 Batch extraction

Batch extraction was carried out in the same way as it was done during the OECD 307 transformation study with ^{14}C labelled compounds described in section 4.2.8. Briefly, 1 g of dry soil was extracted overnight and centrifuged successively with each of three different solvents in a solid to liquid ratio of 1:2. The three extracts were combined and filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

3.2.3.3 Ultrasonic solvent extraction

1 g of dry soil was extracted for 10 min in an ultrasonic bath *(SONOREX DIGITEC DT 514 H | BANDELIN)* at room temperature with 10 mL of the ternary solvent mixture composed from methanol/acetone/water (50/25/25 (v/v/v)). The supernatant was decanted after 10 min centrifugation at 2000 rpm. Extraction and centrifugation was repeated twice and the extracts were combined and filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

3.2.3.4 Microwave assisted solvent extraction

1 g of dry soil was weighed into a glass fibre filter, placed into the extraction vessels and 20 mL of the ternary solvent mixture composed from methanol/acetone/water (50/25/25 (v/v/v)). A 10 min temperature ramp to 160 °C was programmed and kept constant for 30 min (MARS6, CEM). After cool down and solvent removal, a second extraction run was performed. Extracts were combined and filled up to 50 mL with Milli-Q water. For LC-MS/MS analysis, 990 µL of the extract were mixed with 10 µL of the internal standard solution.

3.2.4 LC-MS/MS analysis

The extracts were measured using LC-MS/MS and quantified using $^{2}H/^{13}C$-labelled internal standard substances (Table 3).

LC-MS/MS measurements were performed with a LC 1260 infinity series system by Agilent Technologies (Waldbronn, Germany) coupled to a Sciex Triple Quad 6500+ mass spectrometer (Darmstadt, Germany). The method applied is based on the methods published by Hermes *et al.* and Brand *et al.* (Brand et al., 2018; Hermes et al., 2018). The LC system consisted of a degasser, binary pump, isocratic pump, autosampler and column oven. Chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 3.5 µm) with a Zorbax Eclipse XDB-C8 Guard Column (2.1 × 12.5 mm, 5 µm), both obtained from Agilent. Solvent A consisted of 0.1 % aqueous formic acid and solvent B was acetonitrile. The binary gradient started at 98 % A for 1 min, decreased to 80 % within one minute. Then A was decreased to 0 % within 14.5 min, which was kept for 2.5 min. Within 0.1 min A was increased to 98 % and this was kept for re-equilibration until the end of analysis after a total of 25 min.

The mass spectrometer was equipped with an electrospray source in switching polarities and detection was achieved in the scheduled MRM mode. For every compound two characteristic MRM transitions were recorded and one MRM transition for each internal standard compound (Chapter A 3.1). Instrument control and data acquisition was performed with Analyst 1.6.3 and peak integration as well as data evaluation with MultiQuant 3.0.2. Recovery experiments were performed by spiking analytes into selected sample extracts followed by LC-MS/MS analysis.

3.3 Data evaluation

The experiments generated about 3780 data points as 42 substances were analysed in triplicates for each of the three soils and for each of the ten different extraction procedures. Due to the large number of individual results, the data was processed to obtain a comparable and conclusive statement.

First, the triplicate concentrations of each compound, soil and extraction procedure were averaged.

To compensate for degradation, the concentrations of each compound in a soil were normalised over the 10 extraction procedures (EP) applied. For that, the apparent extraction efficiency (AEE) for compound c in soil b using extraction procedure i was calculated:

$$AEE(c,b)_i = \frac{C(c,b)_i}{[\![\max(C(c,b)]\!]_{i=1,\dots,10}} \ [\%]$$

c = compound

b = soil

i = extraction procedure

$C(c,b)_i$ = concentration of compound c in soil b using extraction procedure i

$\max(C(c,b)_{i=1,\dots,10}$ = maximum concentration of compound c in soil b within all 10 extraction procedures applied

Thus, the AEE is 100% for that EP yielding the highest concentration of compound c in soil b within all 10 extraction procedures, while AEEs of the other EPs for compound c in soil b were ranging between 0 and 100 %.

Afterwards, the AEEs obtained for the three soils applied were averaged for each compound and each extraction procedure gaining the soil averaged AEEs (SAAEE). The SAAEEs for each set of 42 compounds obtained for a specific extraction procedure were used to calculate the upper and lower quartiles as well as the median. Box plots were generated with using Origin 8.1 (Table A6). Since 42 compounds were evaluated in total, every quartile represents approximately 11 compounds. The range from the minimum to the beginning of the box (first quartile) implies 25 % of the compounds showing the lowest apparent extraction efficiencies. The box frames 50 % of the compounds around the median apparent extraction efficiencies. The range from the upper limit of the box (fourth quartile) until the maximum represents those 25 % of the compounds showing the best apparent extraction efficiency for this respective extraction procedure. Since 42 compounds were evaluated in total, every quartile represents approximately 11 compounds.

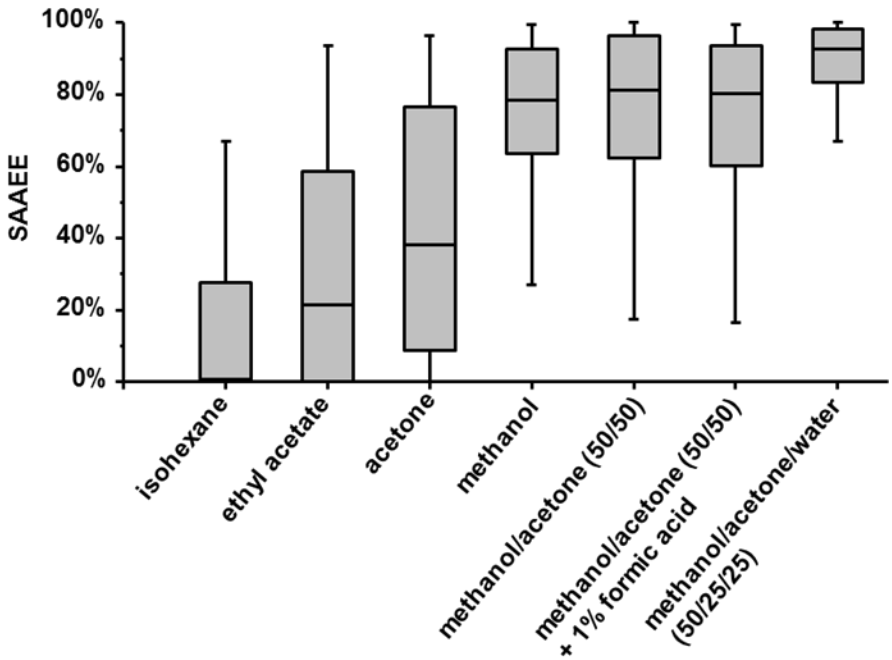
3.4 Results and discussion

A comparative study was launched to achieve an extended overview about the efficiency of several extraction methods for a large variety of organic compounds and different soil types. A set of 42 compounds was spiked to the three selected soils. The substances chosen represent a wide range of polarity (log K_{OW}-value), chemical structures and substance classes such as pharmaceuticals, pesticides and biocides.

Figure 5 displays the box-plot graphs of the soil averaged apparent extraction efficiencies (SAAEEs) for seven different extraction solvents tested for PLE. The solvents are shown with increasing polarity from left to right. The boxes group the SAAEEs of the substances in their quartiles. The ternary mixture consisting of methanol/acetone/water (50/25/25, v/v/v) attained the highest SAAEEs and the lowest statistical variations (<20 %). The median of the SAAEE was 94 %. About 75 % of the substances tested showed a SAAEE of more than 83 %.

Poor extraction efficiencies were obtained with the nonpolar solvent iso-hexane, where only half of the compounds showed a SAAEE > 1 %. Using ethyl acetate and acetone median values of 21 and 39 % were achieved, respectively, accompanied by a high statistical variation. The extraction solvents methanol, methanol/acetone as well as acidified methanol/acetone showed very comparable results for the selected 42 compounds with a median of about 80 %. This points out that neither the addition of acetone nor the acidification significantly alone sufficiently improved the extraction efficiencies and their variability.

Figure 5: Comparison of the soil averaged apparent extraction efficiencies (SAAEE) of seven different extraction solvents tested for PLE



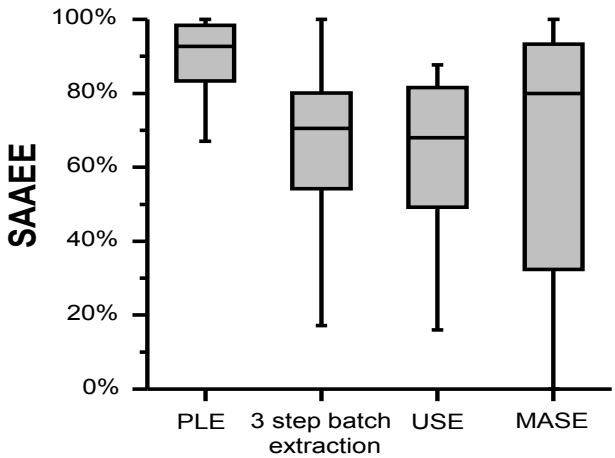
Reference: Federal Institute of Hydrology (2019)

A significant improvement of the SAAEEs was observed by the addition of water to the organic solvents. Comparing the results for methanol/acetone and methanol/acetone/water the median increased from 82 to 94 % and the difference between the first and the third quartile decreased from 32 % to 13 %.

In comparison to the extraction mixtures without water, a significant improvement of the SAAEEs was observed for the ternary mixture containing water possibly due to widening of soil pores by swelling the clay particles (Ferrer and Furlong, 2002; Hawthorne et al., 2000; Vazquez-Roig and Picó, 2015).

After identifying the optimal extraction solvents, different extraction techniques were compared. Figure 6 shows the boxplot graphs of the SAAEEs of four different extraction methods (PLE, USE, MASE, 3SBE). The extractions were carried out using always the same solvent mixture (methanol/acetone/water (50/25/25, v/v/v)), except for the three- step batch extraction (3SBE), in which three different solvents were consecutively applied (0.01 M aqueous $CaCl_2$, methanol/water (50/50, v/v) and methanol/acetone (50/50, v/v)).

Figure 6: Comparison of the soil averaged apparent relative extraction efficiencies (SAAEE) of four extraction techniques with methanol/acetone/water (50/25/25, v/v/v) as extracting agent



Reference: Federal Institute of Hydrology (2019)

PLE showed the highest recoveries with a median of 94 %, and the smallest difference between the first and the third quartiles of 13 % (Figure 6). Ultrasonic extraction (USE) and 3SBE exhibited comparable results with a median of about 70 %, but with differences between the first and the third quartiles between 32 and 26 %. The microwave assisted solvent extraction (MASE) achieved a value of 81 % for the median of the SAAEEs, but a wide difference between the first and the third quartiles of 61 %, possibly related to thermal degradation of analytes at 160 °C.

Despite the fact that several of the spiked compounds are prone to transformation in the soil spiking experiments, more than 75 % of the initial concentration was recovered after PLE for about 27 of the 42 spiked compounds, highlighting the elevated extraction efficiency obtained by PLE with the ternary extraction mixture at 100°C and 100 bar (Chapter A3.2.2).

3.5 Conclusions

It can be concluded, that PLE is a widely used and an automatable technique commonly accepted for the extraction of organic substances from soil and other solid materials such as sediments. The extracts obtained can frequently be injected directly into LC-MS/MS and in some cases into GC/MS. When necessary, a sorbent can be added into the PLE extraction cell for in-cell clean-up to remove impurities disturbing the measurements (Abdallah et al., 2013; Abdul et al., 2017; Cocco et al., 2011; Haglund and Spinnel, 2010; Negreira et al., 2011; Pintado-Herrera et al., 2014, 2016; Schantz, 2006; Vallecillos et al., 2012; Vazquez-Roig and Picó, 2015).

Hence, the results of this study highlights that pressurised solvent extraction at 100 °C and 100 bar with the ternary solvent methanol/acetone/water (50/25/25, v/v/v) provided elevated extraction efficiencies (SAAEEs) with an acceptable uncertainty for a broad spectrum of organic chemicals and three different soils. It should be noted that the extraction results were widely independent of the compounds spiked and the soil types selected.

As consequence, it can be suggested to use this extraction procedure for the analysis of organic chemicals in soil during registration.

Although the PLE conditions used were applicable for a wide range of organic substances, it cannot be excluded that for specific compounds the used PLE conditions might be not optimal, for instance due to thermolability or other properties. As a consequence, the applicability of the PLE procedure needs to be confirmed for each compound and each soil matrix studied. However, we assume that for the majority of organic substances the PLE conditions suggested are at least a good starting point for further optimization of the extraction procedure.

4 Transformation experiments according to OECD guideline 307

A main task of this project was to determine and characterise NER after transformation experiments according to OECD guideline 307 with three different test compounds and three soils. The NER were characterised following the scheme developed by Eschenbach and Oing (Eschenbach and Oing, 2013b). In the transformation studies ^{14}C-ring-labelled test substances were applied. The radiolabel allowed to elucidate the fate of the spiked compounds including formed NER.

As described in chapter 2.2, triclosan, fenoxycarb, and acetaminophen were selected representing different substance classes as well as different degradation behaviour and formation of different types of NER. After an incubation period of up to 100d the soils were sequentially extracted with different procedures obtaining radioactivity related to NER fractions in the soil matrix. Afterwards the remaining NER containing soil was further extracted/treated to get deeper insights about the types of bound residues. The experimental approach and the attained results of the transformation experiments and of the different extraction/treatment procedures are discussed in this chapter.

4.1 Materials

4.1.1 Chemicals

The ^{14}C-labelled test compounds were obtained from Hartmann Analytic (Braunschweig, Germany) (Table A12). All other used chemicals are given in Chapter A 4.1.2.

4.1.2 Laboratory equipment

The materials used are given in Chapter A 4.1.1.

4.2 Experimental

4.2.1 Test setup

Aerobic transformation experiments with ^{14}C-labelled compounds were carried out following the OECD guideline 307 for the transformation of chemicals in soil. In total nine transformation experiments were performed, using the ^{14}C-ring(U)-labelled test substances triclosan (TCS), fenoxycarb (FEC) and acetaminophen (ACT) which were spiked to the three standard soils Lufa 2.2, Lufa 2.3 and Lufa 2.4.

The standard soils Lufa 2.2/2.3/2.4 were obtained from the ‚Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer' (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, 2016). To obtain a crumbly soil structure which allowed both, a good handling and a proper ventilation of the soil, the soil moistures were individually adjusted for each soil. The moisture chosen for each soil, as well as its maximum water holding capacity are summarised in Table 4. According to OECD guideline 307 the moisture of the soils had to be 40–60 % of WHC_{max}, which was achieved for all three soils. After moisture adjustment, the soils were pre-incubated for two weeks prior to the test start.

Each of the experiments consisted of eight parallel test setups which were successively sacrificed for sampling during the experiments. Each parallel test setup (test string) consisted of an amber glass test vessel containing the spiked test soil and several sealed glass vessels, which were connected in series with stainless steel cannulas and polyurethane tubings as illustrated in Figure 7. Overall, 72 test strings were prepared and sampled after the respective incubation time. The three experiments of each radiotracer compound were always conducted in parallel.

By using a vacuum pump a slight but steady flow of ambient air was sucked through the entire test string, which was regularly adjusted with throttle check valves (Table A8; Table A17). At first, the air was humidified before reaching the test vessel, protecting the soil from drying out. The air was then led through a safety vessel, preventing backflow before passing the trap for organic volatiles filled with paraffin. After passing a second safety vessel, the air was finally led through the $^{14}CO_2$-trap filled with

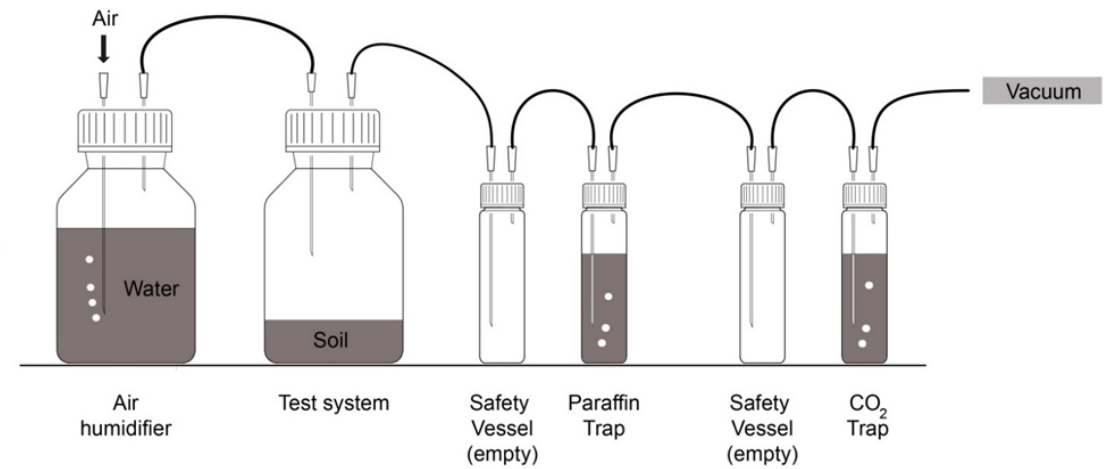
5 M sodium hydroxide solution and thiazole yellow G (Table A12) as indicator to prevent exhaustion of the trapping agent. Figure 8 shows a photo of the test setup of fenoxycarb for each soil type after 4 d.

Table 4: Soil moisture used for the transformation test following OECD guideline 307 (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, 2016)

Soil	Selected moisture	WHC_{max} (g/100 g)	Percentage of WHC_{max*}
Lufa 2.2	20 %	44.6	44 %
Lufa 2.3	15 %	35.6	42 %
Lufa 2.4	25 %	44.8	55 %

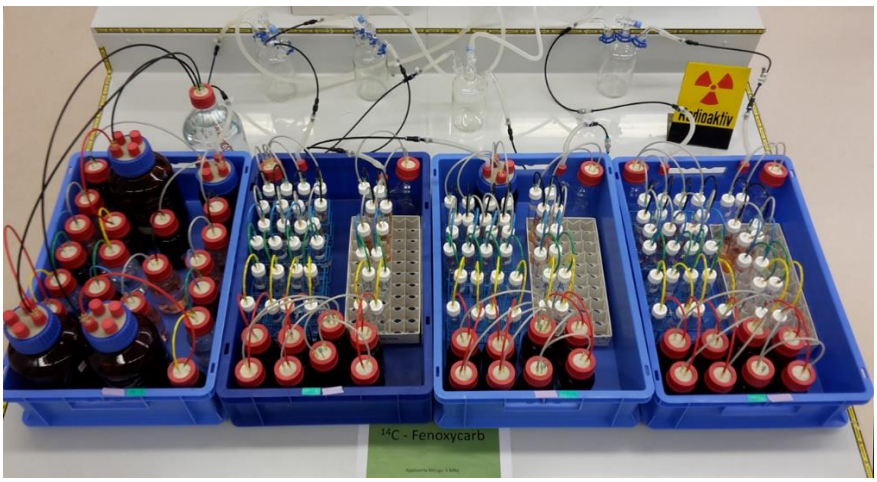
Reference: Federal Institute of Hydrology (2019) and Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Analyses Data Sheet for Standard Soils according to GLP. 2016

Figure 7: Scheme of the applied experimental setup with the soil containing test system



Reference:Federal Institute of Hydrology (2019)

Figure 8: Photo of the experimental test setup for the OECD test 307 with fenoxycarb[4]



Reference: Federal Institute of Hydrology (2019)

[4] The left box contains three test systems with 1 kg soil each and the paraffin and CO_2 traps connected to each test system. The other three boxes contain test systems with 100 g soil and paraffin and CO_2 traps separated for each test system. The first two test systems were already sampled after 1 and 4 days of incubation, respectively.

4.2.2 Spiking of the test substances and homogenization of the spiked test soils

Obtaining a homogenous distribution of organic chemicals spiked to moist soils is a challenging issue (Fent et al., 2003; Girardi et al., 2011; Höltge and Kreuzig, 2007; Northcott G. L. and Jones K. C., 2000; Northcott and Jones, 2000a). The following procedure was applied for obtaining a homogenous distribution of each of the spiked organic chemicals in the three soils. First, portions of 50 g of the different dry ground soils were spiked with 2 MBq of the test compounds triclosan (0.25 mg in 4 ml acetone) or fenoxycarb (0.43 mg in 4 ml acetone) or acetaminophen (0.11 mg in 4 ml methanol).

The spiked soils were homogenised for 30 min using an overhead shaker. Afterwards, the solvent was allowed to evaporate at room temperature before the spiked soil was thoroughly mixed with 450 g of the corresponding moist soil with a handheld electric mixer. Then, 1500 g of the corresponding moist soil were added successively and were homogenised. The moist soil was stirred for at least 30 min, leading to about 2 kg of spiked and homogenised soil.

The proper homogenization was confirmed by combustion analysis of multiple soil aliquots.

Figure 9: Photo of homogenised soil (Lufa 2.2) spiked with fenoxycarb[5]

Reference: Federal Institute of Hydrology (2019)

After verification of a proper homogenisation, soil portions of 100 g were filled into seven test amber vessels and one portion of 1000 g soil into one large test vessel, respectively.

The soil was incubated at room temperature under exclusion of light. During the incubation period, the steady gas flow and the tightness of the system was regularly controlled and adjusted.

4.2.3 Sampling procedure

Each individual test setup was destructively sampled after defined incubation periods (Table 5). In total, 72 test setups were used. No significant loss of moisture was observed over the incubation time. After incubation, the respective setups were disconnected from the vacuum and all connecting tubings were removed. Then, the mass of the test vessels, the sodium hydroxide and the paraffin traps were measured and radioactivity was measured in the gently homogenised soil, and in both traps. Three three aliquots of 15 g incubated soil were subjected to three-step batch extractions (3SBE) to quantify the extractable and non-extractable fractions. A soil aliquot of 10 g was subjected to moisture quantification. All soil aliquots were stored frozen until analysis. Additional details on the radiochemical analysis are provided in the Supplementary Information.

[5] In total, 2 kg moist soil were homogenized for each test compound and test soil combination and apportioned into the eight vessels (100 g) and one vessel (1 kg), respectively.

Table 5: Summary of the prepared test strings and the scheduled sampling

Compound	Soil type	Scheduled sampling times [days after spiking]								
Triclosan	Lufa 2.2	0	1	4	7	14	20	34	60	100
	Lufa 2.3	0	1	4	7	14	20	34	60	100
	Lufa 2.4	0	1	4	7	14	20	34	60	100
Fenoxycarb	Lufa 2.2	0	1	4	11	15	21	35	60	100
	Lufa 2.3	0	1	4	11	15	21	35	60	100
	Lufa 2.4	0	1	4	11	15	21	35	60	100
Acetaminophen	Lufa 2.2	0	1	2	5	8	12	16	21	35
	Lufa 2.3	0	1	2	5	8	12	16	21	35
	Lufa 2.4	0	1	2	5	8	12	16	21	35

Reference: Federal Institute of Hydrology (2019)

4.2.4 Determination of the water content in soil

The water content of each sample after incubation was determined after sampling by weighing before and after lyophilisation of a soil aliquot.

4.2.5 Determination of the total radioactivity in soil

The total radioactivity in the soils was determined at least in triplicate by combustion of defined lyophilised soil aliquots using a sample oxidiser Model 307 loaded with Carbo Sorb E which collected the $^{14}CO_2$ formed during combustion and mixed with the scintillation cocktail Permafluor E+. The resulting samples were analysed for ^{14}C radioactivity using a Tri-Carb 2800 TR. Reliable functionality of the oxidiser was ensured by daily recovery and memory effect experiments using a ^{14}C Spec Check standard following the instruments' manual (see Table A9-A20).

4.2.6 Determination of radioactivity in sodium hydroxide-traps

Radioactivity in the sodium hydroxide traps was measured in order to determine trapped $^{14}CO_2$ during the degradation experiment. For that a weighed aliquot of sodium hydroxide was mixed with Hionic-Fluor scintillation cocktail and analysed for ^{14}C radioactivity using the Tri-Carb 2800 TR (see Table A9-A20).

4.2.7 Determination of radioactivity in paraffin-traps

The non-polar volatile transformation products trapped in paraffin were analysed for ^{14}C radioactivity by mixing a weighed aliquot with Ultima Gold scintillation cocktail before analysing ^{14}C radioactivity using a Tri-Carb 2800 TR (see Table A9-A20).

4.2.8 Three-step batch extraction (3SBE) and determination of radioactivity in soil extracts

After incubation soil samples were subjected to three-step batch extraction (3SBE). For that, sub-samples of 15 g moist soil were extracted in triplicate.

In the first extraction step 15 g soil were shaken sample for 24 h using a horizontal shaker at 200 rpm with aqueous 0.01 M $CaCl_2$ solution with a solid to liquid ratio of 1:2. After centrifugation at 2000 rpm for 7 min the supernatant was decanted. The supernatant was analysed for ^{14}C radioactivity by mixing a weighed aliquot of about 1 g with 5 mL Ultima GOLD scintillation cocktail and measured using the Tri-Carb 2800 TR (see Table A9-A20).

The second extraction step was accomplished using methanol/water (50:50, v/v) as extracting agent applying the same conditions as described above. Accordingly, acetone/methanol (50:50 (v/v)) was used in the third extraction step. FloScint III scintillation cocktail was used for ^{14}C measurements of the second and third extraction step, in which about 1 g extract was mixed with 7 mL cocktail.

After extraction, the remaining solvent in the soil was allowed to evaporate prior to homogenization and determination of the remaining total ^{14}C radioactivity in the extracted soil.

4.2.9 Pressurised liquid extraction

Dried soil (e.g. after 3SBE) was weighed into the extraction cell. Extraction pressure was set to 100 bar and the temperature set to 100 °C (Speedextractor 914, Büchi). Three consecutive extraction cycles with 15 min each were performed followed by flushing with solvent. Discharge time was set to five minutes. A ternary solvent mixture composed from methanol/acetone/water (50/25/25 (v/v/v)) was used (see Chapter 3). The extraction cell was filled to capacity according to the following order: a glass fibre filter was placed on the bottom of a 40 mL extraction cell, followed by a cellulose filter, and 5 g of dried sample, followed by another cellulose filter. Any empty volume was filled with glass beads (2 mm diameter) (Table A19).

4.2.10 Radio-HPLC analysis of soil extracts

After determination of the total radioactivity in soils and soil extracts, the extracts of batch extraction and PLE were analysed by Radio-HPLC (Table A9), determining thus the percentages of the parent compound, as well as known and unknown transformation products in a sample. It was necessary to concentrate the extracts to improve the limit of quantification (LOQ).

For the 3SBE of triclosan, the extracts from extraction step 2 and 3 of one sampling date were combined, since the extracts of step 1 contained negligible amounts of radioactivity, corresponding to <1 % of the initially applied radioactivity. The organic solvent in the combined extracts was allowed to evaporate in a fume hood at room temperature. The aqueous extract was decanted in a vial and radioactivity adsorbed to the inner surface of the extraction vessels, was rinsed down using 5 mL of methanol. The methanol was combined with the aqueous extract and these extracts were then subjected to determination of total radioactivity by LSC and to Radio-HPLC analysis.

The extracts from 3SBE of fenoxycarb were combined in the same way. After evaporation of the organic solvent content in the fume hood, the aqueous residue was lyophilised. The residue was re-dissolved in 3 mL methanol/water (50/50 (v/v)) and subjected to LSC determination for total radioactivity Radio-HPLC analysis.

Radio-HPLC analyses of soils incubated with acetaminophen were not possible, since the radioactivity extracted was below LOQ.

The Shimadzu HPLC system consisted of a CTO-10ASvp column oven, a DGU-14A degassing unit, two LC-10ADvp pumps, a SPD-10Avp UV-Vis detector, a SIL-10ADvp auto-injector and a CBM-20A communication bus module (all Shimadzu Europe Ltd., Duisburg, Germany). A Zorbax Eclipse XDB-C8

(C8-modified silica, end-capped) 4.6 × 150 mm, 5-µm HPLC-column (Agilent Technologies Deutschland GmbH, Böblingen, Germany) protected by a Chromolith Guard column RP-18e (Merck KGaA, Darmstadt, Germany) was used. Oven temperature was set to 40 °C, injection volume was 500 µL. As mobile phase A 0.1 % aqueous formic acid and B acetonitrile were used.

The gradient programme was as follows: the percentage of acetonitrile was raised from 70 to 90 % in 30 min, lowered to 70 % in 1 min and kept at 70 % for 4 min. Flow rate was set to 1 mL/min. For determination of radioactivity, the HPLC system was equipped with a Radiomatic 610TR Flow Scintillation Analyser (PerkinElmer Deutschland GmbH, Rodgau, Germany). Radio-chromatograms were processed using the FLO-ONE radio-detector Software (PE). Scintillation cocktail flow (FloScint III, PE) was set to 2 mL/min.

4.2.11 Additional experiments for the characterisation of PLE_NER

4.2.11.1 Silylation

To analyse the NER fraction physically entrapped remaining in the soil after PLE (PLE_NER), silylation was performed in triplicate, similar as described elsewhere (Dec et al., 1997b; Haider et al., 1992; Haider et al., 1993). For that, 1 g of soil was mixed with 10 mL acetone and 1 mL of trimethylchlorosilane was added. The mixture was shaken overnight on a horizontal shaker (200 rpm) and centrifuged for 7 min at 2000 rpm. Afterwards, the radioactivity in the supernatant was determined by mixing a weighed aliquot of 0.5 g with 15 mL Ultima GOLD scintillation cocktail (PE) and counting for 10 min using the Tri-Carb 2800 TR (Table A16).

4.2.11.2 EDTA-Extraction

Additionally, the physically entrapped fraction remaining in soil after PLE was analysed by EDTA extraction in triplicate as an alternative method for silylation. The procedure applied was similar to one applied by Eschenbach et al. (Eschenbach et al., 1998). In detail, 1 g of soil was mixed with 10 mL aqueous 0.1 M EDTA solution (pH 8) (Table A16), shaken overnight on a horizontal shaker (200 rpm) and centrifuged for 7 min at 2000 rpm. Afterwards, the supernatant was decanted and the radioactivity was determined by mixing a weighed aliquot 0.5 mL with 5 mL Ultima GOLD scintillation cocktail (PE) and counting for 10 min using the Tri-Carb 2800 TR.

Organic compounds, which were sorbed to soil after EDTA treatment, were extracted from the soil. For that, 10 mL of methanol/acetone/water (50/25/25 (v/v/v)) were added, shaken for 2 h on a horizontal shaker and centrifuged, as described above. Radioactivity in the supernatant was determined by mixing a weighed aliquot with 5 mL Ultima GOLD scintillation cocktail (PE) and counting for 10 min using the Tri-Carb 2800 TR. The radioactivity from both EDTA treatment steps was summed up and represents the fraction mobilised by EDTA extraction.

4.2.11.3 HCl-Hydrolysis

The pre-extracted (PLE-NER) soils were treated with 6 M hydrochloric acid overnight under reflux in duplicate (Table A10; A15). After cooling down the supernatant was decanted and radioactivity in the supernatant was determined. The remaining soil was additionally extracted with methanol, to quantify the entire radioactivity released by HCl treatment. The extracted radioactivity in the two liquid phases a) HCl and b) methanol were determined by LSC and summed up. The hydrolysate was extracted using liquid-liquid extraction (LLE) with heptane to quantify the lipophilic fraction released by HCl treatment.

4.2.12 Mass balance determination, error analysis and calculation of degradation times

Mass balances of radioactivity were calculated, using the radioactivity initially spiked (C_0) for comparison. For error analysis the Gaussian propagation of uncertainty was used. The software Cake 3.3 (Tessella) was applied to calculate degradation times using data for transformation of the parent compounds (triclosan/fenoxycarb) and the transformation products methyl-triclosan (Me-TCS) and

hydroxyl-fenoxycarb (FEC-OH). Concentrations of the parent compounds at d 0 were set to 100 %. As degradation processes most likely occurred during freezing/thawing and during the 24 h of shaking with aqueous $CaCl_2$ (first step of 3SBE), the concentration data measured in the soil samples of d 0 were considered in the modelling calculations as values after 1 d of incubation. The calculations of DT50 values were conducted using kinetic models for Single First Order (SFO), Double First-Order in Parallel (DFOP), Hockey Stick (HS) and First-Order Multi-Compartment (FOMC) to find the best fit kinetics (FOCUS, 2006).

4.3 Results and discussion

Transformation experiments were performed following OECD guideline 307. Soils were spiked with ^{14}C-labelled compounds and thoroughly homogenised. After the dedicated incubation time the soil was sampled and radioactivity in soil and the various trap materials was determined. Afterwards, a batch extraction of the soils was performed to determine the extractable as well as the non-extractable fraction. Additionally, PLE extraction and HPLC analysis of selected extracts were performed, as well.

In the following sections the results for triclosan, fenoxycarb and acetaminophen are presented, described, compared to earlier studies, and comprehensively discussed.

4.3.1 Triclosan

After spiking, radioactivity related to ^{14}C-triclosan was always quantitatively recovered over the duration of the experiment, with a ^{14}C-recovery ranging between 91 and 107 % (Figure 10). No significant loss of moisture was observed over the incubation time. The only exception was the soil sample of Lufa 2.3 day 14 which was discarded since it dried out due to a leakage.

In all cases volatile species, which were trapped in the paraffin traps were <1 % of applied radioactivity and were considered negligible. Consequently, these volatile fractions are neither shown in the following figures, nor will these be discussed in more detail.

Mineralisation into $^{14}CO_2$

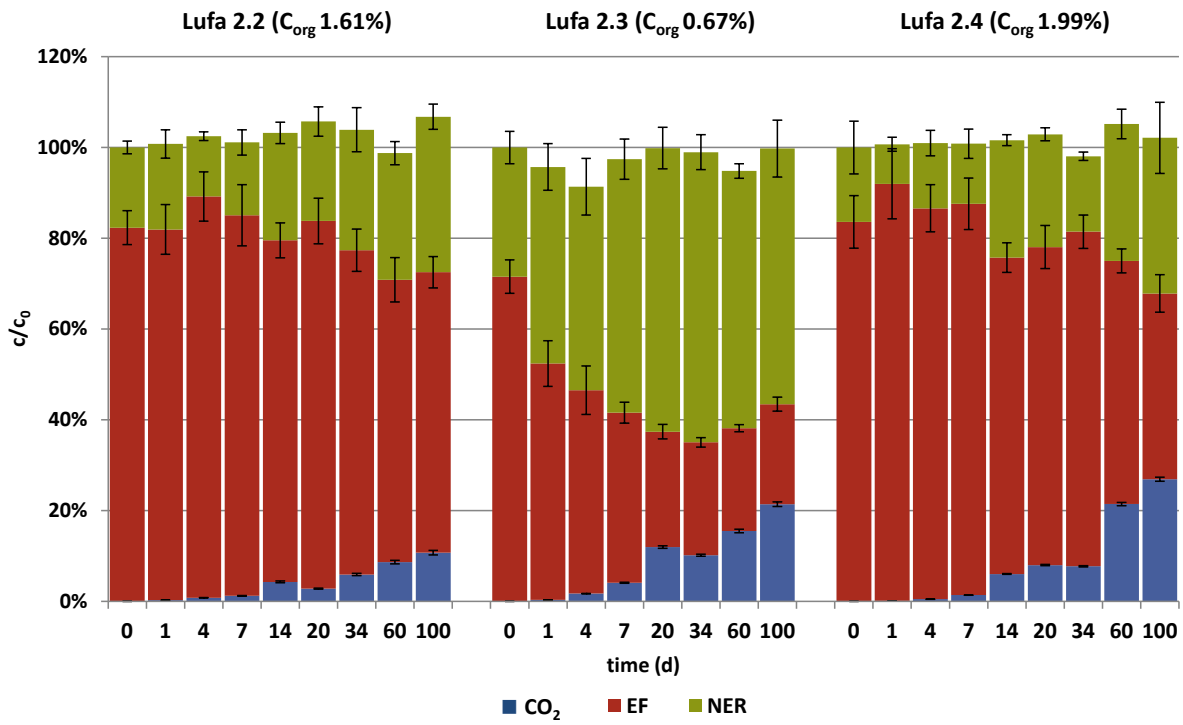
In all three soils the percentage of evolving $^{14}CO_2$ increased continuously (Figure 10). After the entire incubation time of 100 d 11 % (Lufa 2.2), 21 % (Lufa 2.3) and 27 % (Lufa 2.4) of the originally applied radioactivity were mineralised to $^{14}CO_2$, which is in general accordance to values reported earlier. Al-Rajab *et al.* found a mineralisation for triclosan of about 7 % within 42 d (Al-Rajab et al., 2009). Another study found a mineralisation of 12 to 20 % after 64 d in soil amended with activated sludge (Samsøe-Petersen et al., 2003) (Table A21–A23).

Characterisation of radioactivity in soil after incubation of triclosan

A three-step batch extraction (3SBE) was used to quantify the extractable fraction (EF) and non-extractable residues (NER) after soil incubation of triclosan. The results showed a formation of NER continuously increasing with incubation time and decreasing extractable fractions (EF) for all three soils (Figure 10). In Lufa 2.2 the EF decreased from 84 % after 7 d to 64 % after 100 d, while NER increased in the same time from 16 % to 34 % of applied radioactivity. For Lufa 2.4 a similar behaviour was observed with 34 % NER after 100 d. For detailed information see chapter A4.2.2.

However, in Lufa 2.3 a significantly higher percentage of NER (56 %) was formed within 100 d of incubation. This might have been influenced, among others factors, by its organic carbon content, which is relatively low in Lufa 2.3 (Table 1). In contrast, Lufa 2.2 and Lufa 2.4, with higher organic carbon contents, may possibly provide a higher supply of soil organic matter for metabolism of soil microbiota, leading thus to a lower transformation and formation of NER in these soils. In comparison, Al-Rajab *et al.* reported a formation of 43 % NER after incubation of triclosan in soil for 42 d (Al-Rajab et al., 2009). In other studies, the fractions of NER were also in the range of these experiments (Butler et al., 2012; Waria et al., 2011; Ying et al., 2007).

Figure 10: Balance of the applied radioactivity in the test system after incubation of triclosan in soils (see Tables A24-A26).



Reference: Federal Institute of Hydrology (2019)

Chemical analysis of the extractable fraction

For chemical analysis of the extractable fraction (EF) of each of the four sampling times selected, the extracts of the second and third extraction step were combined, concentrated, and analysed by radio-HPLC. The parent compound triclosan was identified and quantified as well as its main transformation product (TP) methyl-triclosan (Me-TCS). The radioactivity, which could not be assigned to these two compounds is summarised hereafter as unknown extractable substances (ES). Figure 11 shows the transformation of triclosan and methyl-triclosan together with the respective ^{14}C mass balance curve for each soil. Although NER for TCS in Lufa 2.2 and Lufa 2.4 were similar, the DT_{50}-value for TCS in Lufa 2.2 of 56 d was significantly higher than those for TCS in Lufa 2.3 and Lufa 2.4, which were between 2.3 d and 3.9 d, respectively (Table 6).

In Lufa 2.2 and Lufa 2.3, the fraction of Me-TCS rose up to 22 % and 16 % of the applied radioactivity within 100 d, respectively. In contrast, Me-TCS peaked after 34 d of incubation at 60 % in Lufa 2.4 and decreases to 34 % after 100 d (Chapter A4.2.4).

Table 6: Degradation times of triclosan and Me-TCS in the respective test soils (calculated with CAKE 3.3)

Soil	DT_{50}^{TCS} (d)	DT_{90}^{TCS} (d)	DT_{50}^{Me-TCS} (d)	χ^2 TCS (%) / χ^2 Me-TCS (%)	Kinetic
Lufa 2.2	56	185	[a]	7.7 / 11.6	SFO
Lufa 2.3	2.3	19	[a]	3.5 / 9.1	FOMC
Lufa 2.4	3.9	23	86	3.8 / 8.5	FOMC

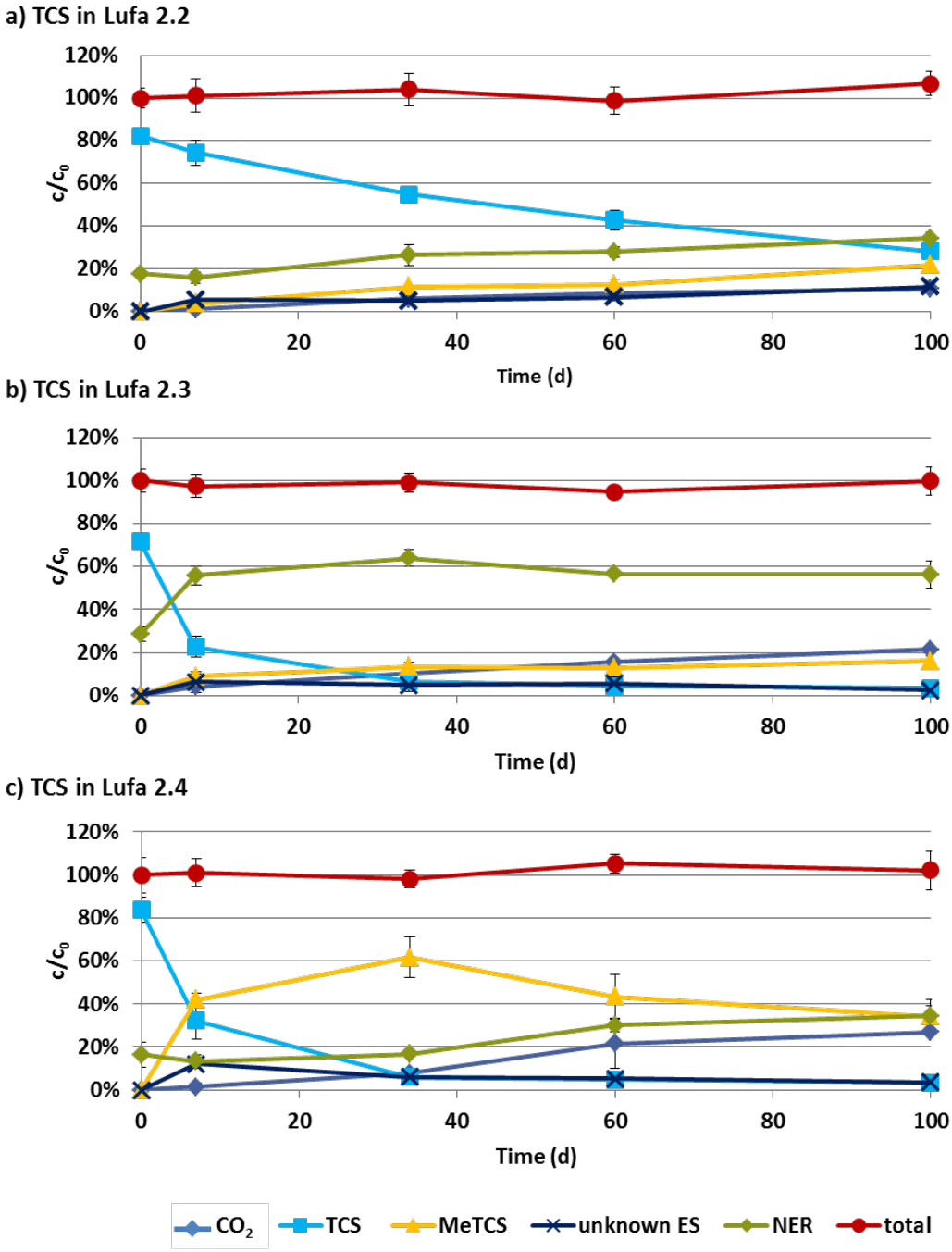
[a] No plausible degradation time could be determined

Reference: Federal Institute of Hydrology (2019)

Ying *et al.* [110] described a half-life for triclosan in aerobic soil of 18 d and 3–35 d were reported by Reiss *et al. and Ying et al.* (Reiss et al., 2009; Ying et al., 2007), both after extraction with methanol and methanol/water. In contrast, Lozano *et al.* described a half-time in soil after bio-solid application of 104 d for triclosan and 443 d for methyl-triclosan (Lozano et al., 2012). However, they extracted the soil under comparably harsh conditions with pressurised liquid extraction at 60°C with water/isopropanol (20/80) as solvent.

The results indicate that the half-life of triclosan is strongly influenced by the extraction procedure applied, due to its strong sorption affinity towards soil.

Figure 11: Fate of triclosan in standard soils expressed as % of applied radioactivity (C_0) (Table A30; A32; A34).



Reference: Federal Institute of Hydrology (2019)

PLE of the NER containing soil

To characterise the NER of triclosan, the soil extracted by 3SBE was extracted further with PLE, which is, in comparison to the 3SBE, an intense extraction method applying elevated pressure and temperature achieving highest extraction efficiencies (chapter 3).

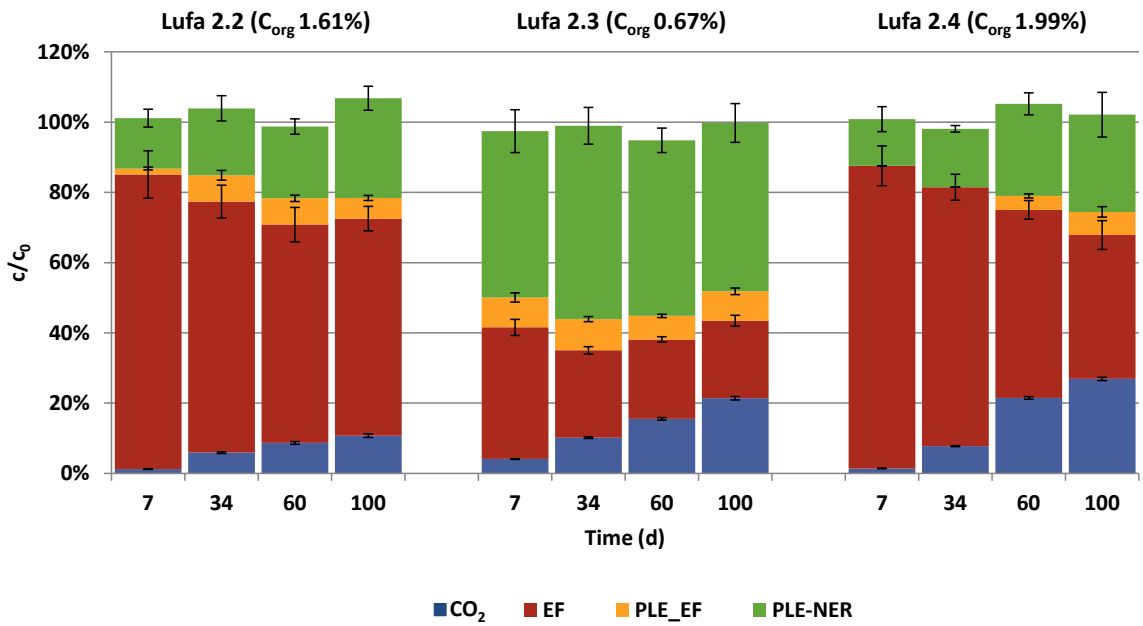
Figure 12 shows that PLE was able to release additional radioactivity from the pre-extracted soils. In comparison to the 3SBE, another 6–8 % of applied radioactivity were extracted by PLE after 100 d of incubation for all soils. Among the three soils, the highest quantity of radioactivity released by PLE was observed for Lufa 2.3, for which the highest quantity of NER was observed.

According to Eschenbach and Oing (2013) the fraction of radioactivity extractable by PLE (after a batch extraction was already conducted) can be attributed to NER-Type 1 (cf. Figure 1), which is assumed to be strongly bound to the soil (Eschenbach and Oing, 2013b). It might be assumed that a significant proportion of the physically entrapped fraction (NER-Type 2 - Eschenbach and Oing) might be contained in the PLE extract too, due to the intense extraction conditions.

At day 7 and 34 no further radioactivity was extracted from Lufa 2.4 using PLE. A reason for this behaviour might be, that TCS and its TPs (e.g. Me-TCS) are initially associated to the soil by a relatively weak binding, still allowing complete extraction by 3SBE. The binding intensity to soil might become stronger with incubation time and a fraction might be extractable using PLE only. However, in the two other soils no similar behaviour was observed.

In the following, the radioactivity remaining in the soil after PLE will be named PLE_NER. This represents the fraction of radioactivity which could not be mobilised even with PLE. For TCS in Lufa 2.2, Lufa 2.3 and Lufa 2.4 PLE_NER fractions of 28, 48 and 28 % of the initially applied radioactivity after 100 d were observed, respectively.

Figure 12: Extractability of TCS by 3SBE and PLE from Lufa standard soils (see Table A39-A41)



Reference: Federal Institute of Hydrology (2019)

Triclosan is a known and well characterised compound extensively used as biocide in various products.

Due to the high lipophilicity of TCS and Me-TCS hardly any radioactivity was released by the extraction with aqueous $CaCl_2$ solution. The majority of radioactivity was mobilised during 3SBE using organic solvents. As the 3SBE was quite intense, only a small fraction of radioactivity was additionally extracted by the following PLE.

It can be concluded, that PLE is the method of choice for highly sorptive compounds, such as triclosan, due to the elevated extraction efficiency (Gan et al., 1999).

4.3.2 Fenoxycarb

After spiking, radioactivity related to ^{14}C-fenoxycarb was quantitatively (104–106 %) recovered within the parallels for each of the 3 soil types.

Due to problems with the vacuum gradient, $^{14}CO_2$ shifted between some of the parallel setups of a soil type. However, as the ^{14}C-balance for the parallel setups of each soil type was closed, the respective fractions of $^{14}CO_2$ for the individual test systems/parallels could be calculated as difference of the remaining radioactivity measured in the soil after incubation and the initially applied radioactivity at test start (Figure 13). Thus, the sum of radioactivity in the various fractions for FEC in the three soils was always 100 % (Figure 13-Figure 15).

In all cases, volatile species generated from ^{14}C-fenoxycarb trapped in the paraffin traps were < 1 % of applied radioactivity and were considered negligible, as also described before (European Commision, 2010). Consequently, these volatile fractions are neither shown in the following figures, nor will these be discussed in more detail. No significant loss of moisture was observed over the incubation time.

Mineralisation into $^{14}CO_2$

In all three soils fenoxycarb was rapidly mineralised and the percentage of evolving $^{14}CO_2$ increased continuously (Figure 13). After the entire incubation time of 100 d between 48 % (Lufa 2.2), 43 % (Lufa 2.3) and 40 % (Lufa 2.4) of the originally applied radioactivity were mineralised to $^{14}CO_2$. However, $^{14}CO_2$ formed within the first 15 d of incubation, accounted for about 66 % of the total quantity of $^{14}CO_2$ observed within 100 d of the experiment for all soils.

Similar values for the mineralisation of Fenoxycarb in soils were reported by other studies. Sullivan reported a mineralisation of 33 % after 12 month (Sullivan, 2010), the EFSA stated a mineralisation of 24–32 % after 88–91 d (European Food Safety Authority (EFSA), 2010) and maximum mineralisation rates between 38.3 % and 46 % have also been reported (European Commision, 2010).

Characterisation of radioactivity in soil

Again, 3SBE was used to quantify the extractable fraction (EF) and non-extractable fraction (NER) after soil incubation of fenoxycarb (Figure 13).

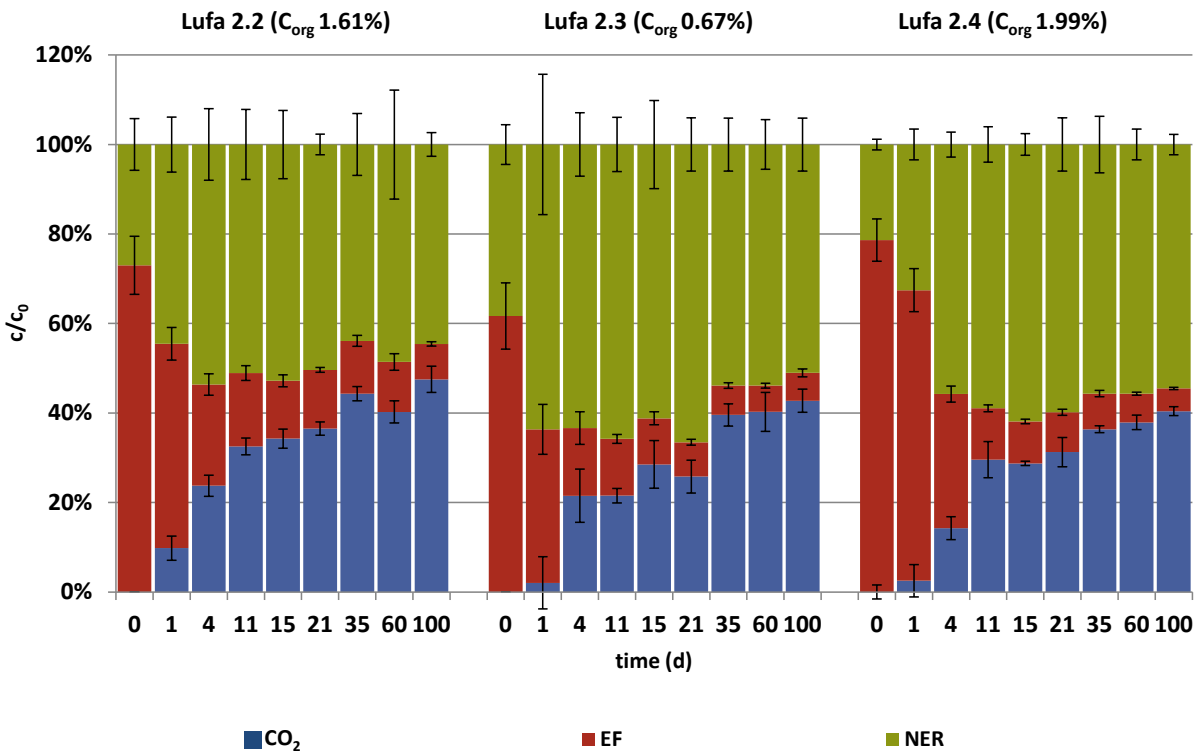
In all soils the quantity of radioactivity extractable using the 3SBE decreased rapidly with increasing incubation time. After 1 d of incubation 34–65 % of applied radioactivity were extracted (EF), whereas after 15 d only 9–13 % and after the full incubation time of 100 d only 5–8 % of the applied radioactivity remained extractable. For Lufa 2.2 the EF decreased from 46 % after 1 d to 8 % after 100 d.

As the EF decreased rapidly, NER were rapidly formed after application of ^{14}C-fenoxycarb and did not change significantly between day 4 and day 100. The behaviour of NER related to fenoxycarb was similar for all three test soils resulting in 45 % NER in Lufa 2.2, 51 % NER in Lufa 2.3 and 55 % in Lufa 2.4 after 100 d.

Observations on EF/NER of this study fit well with results reported earlier (European Food Safety Authority (EFSA), 2010), where NER related to ^{14}C-fenoxycarb were quantified with 53–63 % of the applied radioactivity after 88–91 d. Furthermore, the observed time-dependence of EF/NER is consistent with a biphasic kinetic reported for the aerobic degradation of fenoxycarb, with a

comparably short primary half-life of about 7 d and a significantly longer secondary half-life of about 80 d (Sullivan, 2010).

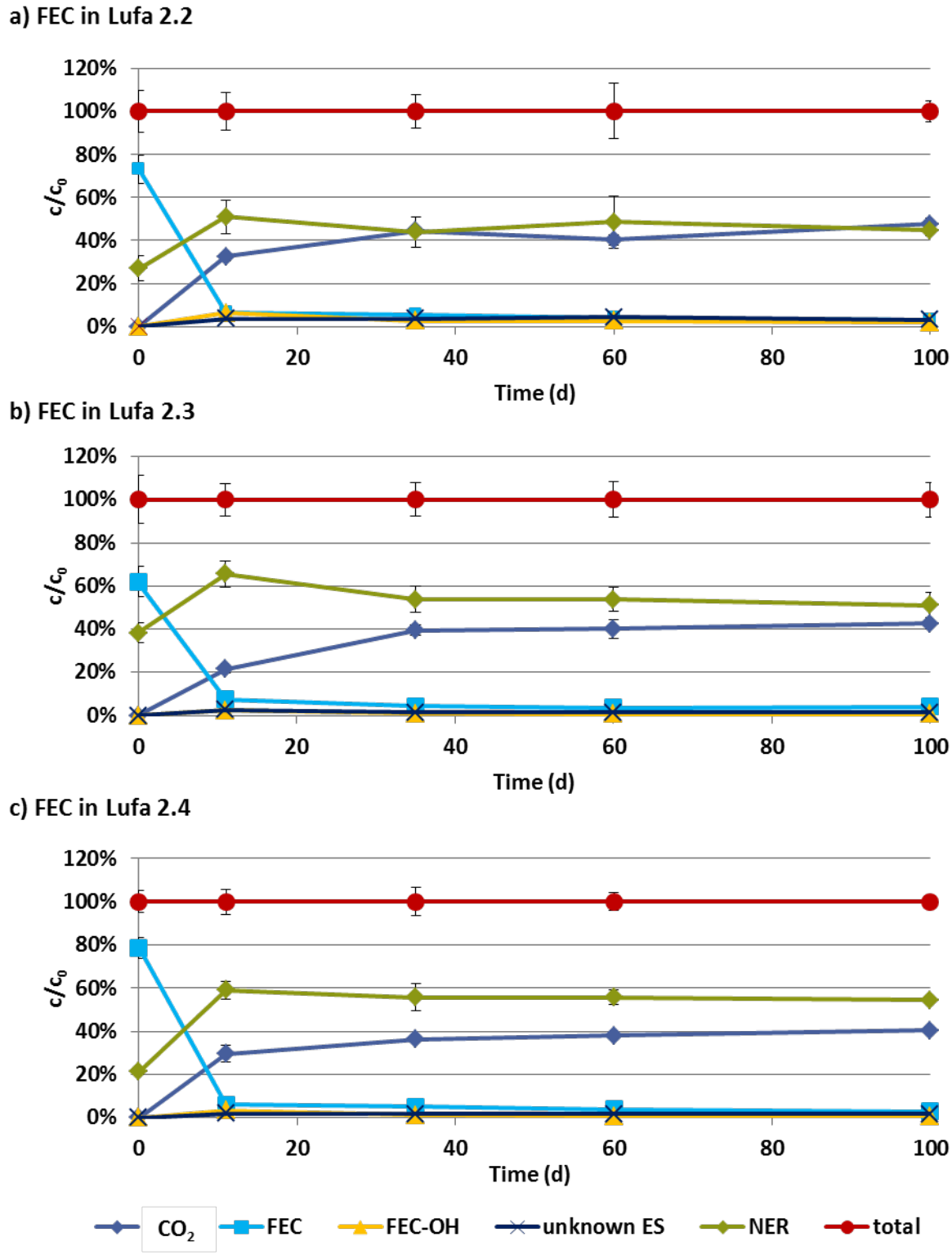
Figure 13: Balance of the applied radioactivity in the test system of fenoxycarb during the incubation time, showing $^{14}CO_2$, extractable and non-extractable fraction (Table A46–A48) [6]



Reference: Federal Institute of Hydrology (2019)

[6] Note: Volatile species were <1% and were therefore omitted from these graphics.

Figure 14: Fate of fenoxycarb in soils (Table A52; A54; A56)



Reference: Federal Institute of Hydrology (2019)

Chemical analysis of the extractable fraction

The parent compound fenoxycarb as well as its transformation product (TP) hydroxy-fenoxycarb were identified and quantified. The radioactivity, which could not be assigned to these two compounds was summarised and assigned as “unknown extractable fraction”.

Figure 14 shows the degradation curve of fenoxycarb and hydroxy-fenoxycarb embedded in the respective ^{14}C mass balances for each soil.

The $DT_{50/90}$ values for fenoxycarb were calculated using the software *Cake version 3.3* (chapter 4.2.12). In all soils ^{14}C-Fenoxycarb was rapidly transformed with DT_{50}-values between 1.6 and 2.8 d and DT_{90}-values between 7.8 and 11 d, as only 6–8 % of the radioactivity initially applied was present as fenoxycarb after 11 d of incubation (Table 7).

The major transformation product was $^{14}CO_2$. At the end of the test 3–4 % of applied radioactivity were present as FEC. For hydroxy-fenoxycarb, the highest percentages observed ranged between 2–6 % after 11 d decreasing down to 1–2 % of the applied radioactivity after 100 d. These results are totally consistent with the biphasic transformation of fenoxycarb, which was reported before (European Commision, 2010; Sullivan, 2010).

In this study, fenoxycarb showed the highest mineralisation rate of the three tested compounds with up to 48 % $^{14}CO_2$. In other studies a mineralisation of 24–32 % was reported for FEC (2010; Sullivan, 2010).

The relatively short half-lives of the active compound fenoxycarb generally agree with literature data, in which a median DT_{50} value of 4 d in dependence on the soil is described. These studies also describe the formation of hydroxy-fenoxycarb as main transformation product in soil being present in minor concentrations below 10 % of applied radioactivity (European Food Safety Authority (EFSA), 2010; Sullivan, 2010) (Chapter A4.3.4).

Table 7: Degradation times of fenoxycarb and FEC-OH in the respective test soils based on the degradation curves

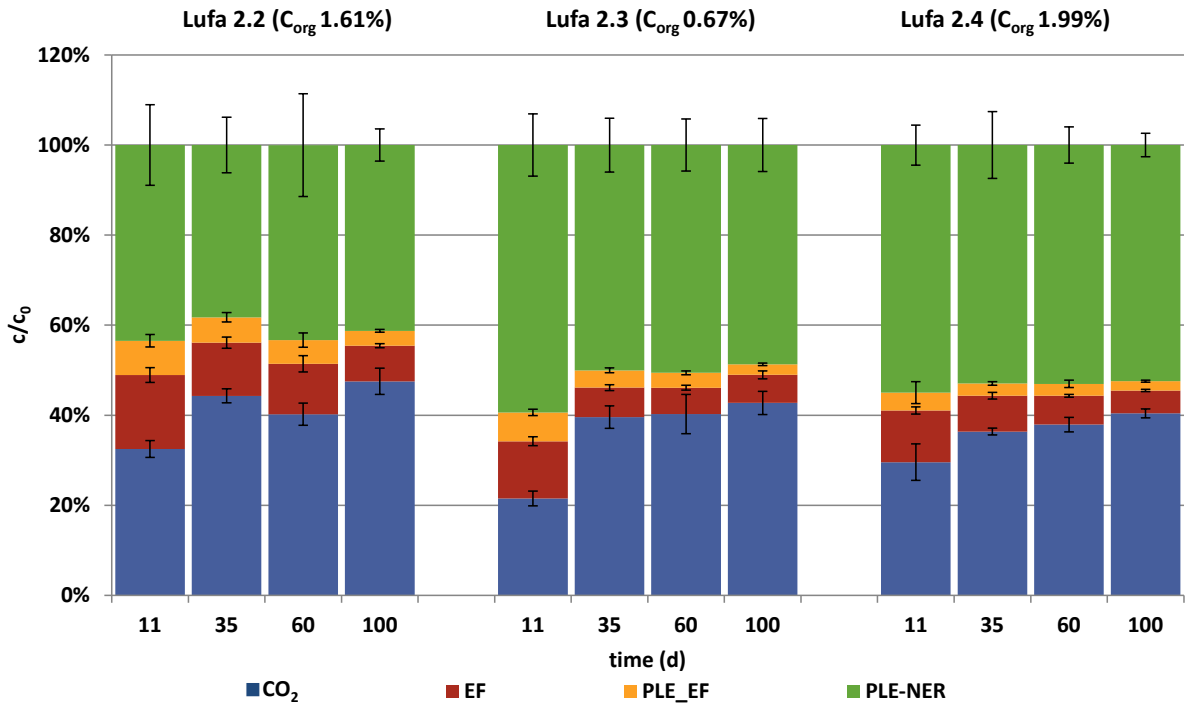
Soil	DT_{50}^{FEC} (d)	DT_{90}^{FEC} (d)	DT_{50}^{FEC-OH} (d)	χ^2 FEC (%)/χ^2 FEC-OH (%)	Kinetic
Lufa 2.2	2.4	7.8	46	8.6 / 14.7	SFO
Lufa 2.3	1.6	11	59	5.7 / 19.7	FOMC
Lufa 2.4	2.8	9.2	9.2	7.1 / 26.6	SFO

Reference: Federal Institute of Hydrology (2019)

PLE of the NER containing soil

To characterise the NER of fenoxycarb the soil was extracted further with PLE.

Figure 15: Extractable and non-extractable fractions in soil after incubation, 3SBE and PLE of fenoxycarb (Table A58-A60).



Reference: Federal Institute of Hydrology (2019)

Figure 15 illustrates the distribution of applied radioactivity after incubation time, 3SBE and PLE. In comparison to 3SBE, minor fractions of 2–3 % of applied radioactivity were extractable by PLE after 100 d, which represent NER-Type 1 according to Eschenbach and Oing (Eschenbach and Oing, 2013b).

Hence, Lufa 2.2, Lufa 2.3 and Lufa 2.4 showed PLE_NER fractions of 41, 49 and 52 % of the applied radioactivity after 100 d, respectively. No significant change in the distribution of the applied radioactivity was observable from day 35 to day 100.

It should be emphasised that the extractability of residues from fenoxycarb was low.

4.3.3 Acetaminophen

The third radio-labelled compound applied was ^{14}C-acetaminophen. In contrast to triclosan and fenoxycarb, a total test time of 35 d was chosen for acetaminophen, since a rapid transformation of acetaminophen in soil and sediment was reported earlier (Li et al., 2014; Loeffler et al., 2005b).

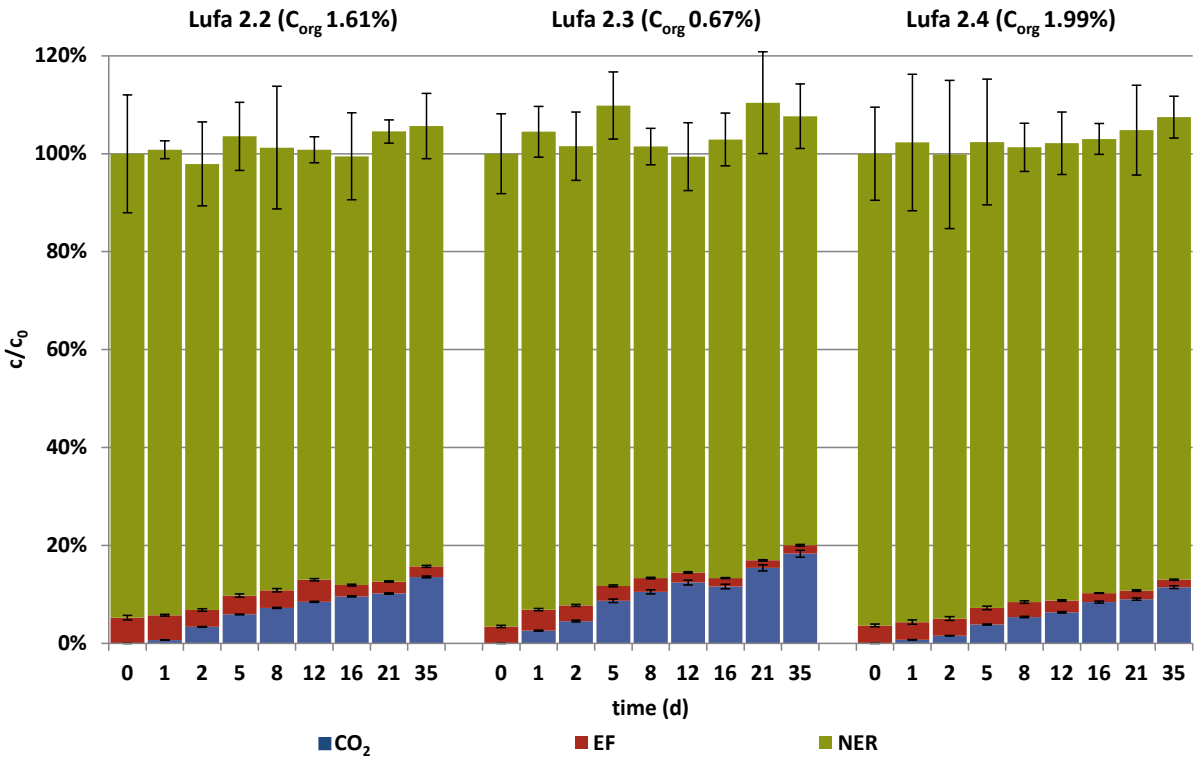
In all cases volatile species trapped in the paraffin traps were < 1 % of applied radioactivity and were considered as negligible. Consequently, these volatile fractions are neither shown in the following figures, nor will these be discussed in more detail.

Regardless of sampling time and soil type, the recovery of the applied radioactivity ranged between 98 % and 110 % showing that ^{14}C-acetaminophen and all its transformation products were quantitatively recovered (Figure 16).

Mineralisation into $^{14}CO_2$

In all three soils the percentage of evolving $^{14}CO_2$ increased continuously (Figure 16). After the entire incubation time of 35 d about 14 % (Lufa 2.2), 18 % (Lufa 2.3) and 11 % (Lufa 2.4) of the originally applied radioactivity were mineralised to $^{14}CO_2$.

Figure 16: Balance of the applied radioactivity in the test system after incubation of acetaminophen (Table A71-A73)



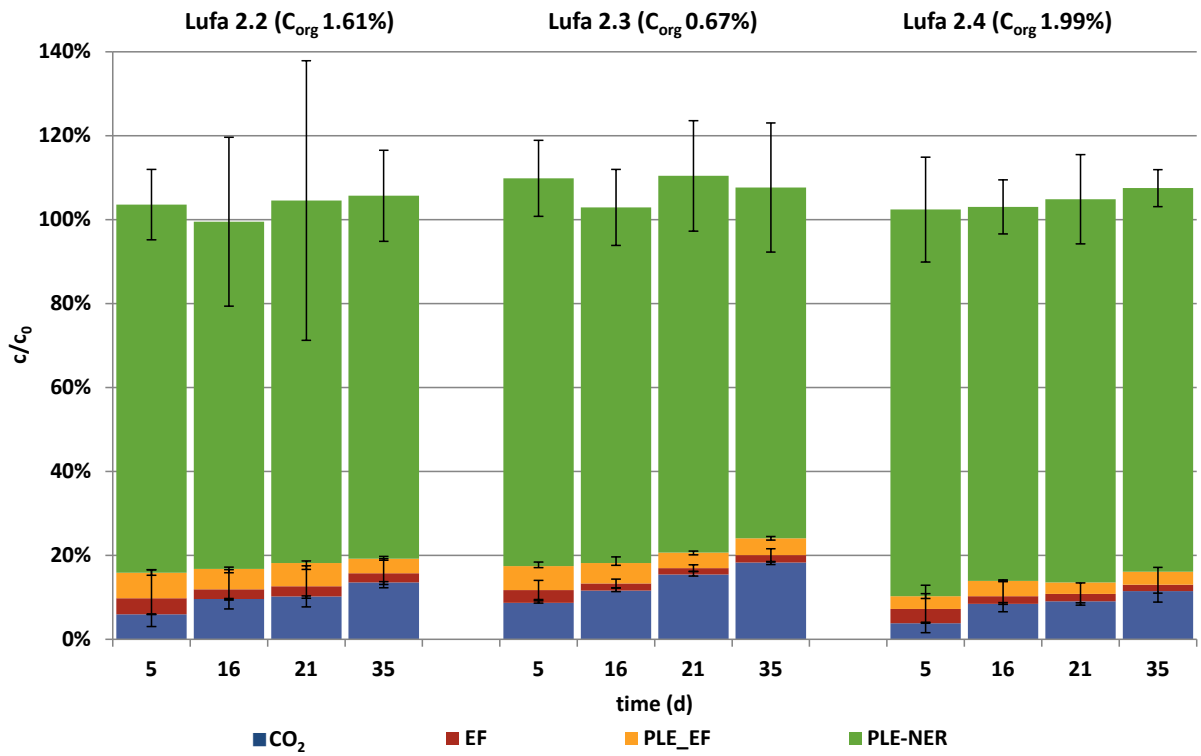
Reference: Federal Institute of Hydrology (2019)

Formation of NER

For all soils, after 35 d of incubation only 2 % of the radioactivity applied as ^{14}C-acetaminophen were extractable using 3SBE (Figure 17). Already directly after test start (d 0) only 3–5 % of the radioactivity were extractable. Complementary, a significant formation of NER was observed immediately after the start of the test. The dynamic of NER formation is widely independent of the soil type since all soils exhibited very similar trends. After 35 d of incubation, between 88–95 % of the applied radioactivity were found as NER.

A chemical analysis of the extractable fraction was impossible, because of the low proportion of extractable radioactivity. However, it is known from literature that a very fast transformation of acetaminophen takes place in contact with soil (Li et al., 2014; Loeffler et al., 2005b).

Figure 17: Extractable and non-extractable fractions in soil after incubation of acetaminophen (Table A77-A79)



Reference: Federal Institute of Hydrology (2019)

PLE of the NER containing soil

To characterise the NER of acetaminophen, the soil was additionally extracted with PLE. The PLE-method used for the extraction of ^{14}C-acetaminophen from soil provided a more exhaustive extraction compared to the 3SBE extraction. After PLE further amounts of only 3–4 % of applied radioactivity were extracted after 35 d of incubation in each of the soils (Figure 17). Fractions of 84–91 % of PLE_NER were found after 35 d. No significant temporal dynamic was observed for the EF, strongly sorbed fraction, and PLE_NER, apart from slightly increasing $^{14}CO_2$ formation.

The mineralisation rate of 11–18 % after 35 d is in good accordance to earlier studies. Li *et al.* observed a mineralisation of 9–17 % after 120 d for different soils (Li et al., 2014). They also observed a rapid and extensive NER formation of 89 % after two days of incubation, which was also observed in our experiments. Due to the rapid and extensive formation of NER, the $DT_{50/90}$-values for all soils were < 1 d, which is in good accordance with other studies (Li et al., 2014; Loeffler et al., 2005a).

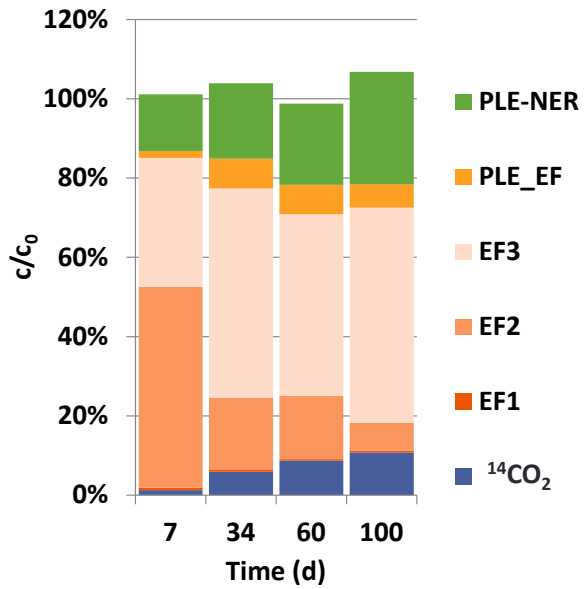
4.3.4 Variability of NER

As stated earlier NER are operationally defined by the extraction procedure used (Barriuso et al., 2008; Gevao et al., 2003; Mordaunt et al., 2005; Northcott and Jones, 2000b). There is no exact and general standard for the extraction procedure, defining where the extractable fraction ends and the non-extractable residue begins. As consequence, for reporting NER data the details of the respective extraction procedure have to be provided.

The relevance of the extraction procedure for the variability of the NER determination was illustrated using our experimental data for TCS, FEC and ACT. 3SBE combines three extraction procedures of increasing intensity to one sequential method. Solvents and duration of the 3SBE were chosen, to provide a high extraction efficiency and hence to limit overestimation of the NER. Additionally, PLE was applied in a fourth extraction step.

As the radioactivity extracted by each step was determined individually, the corresponding NER fractions can be compared for all 4 extraction steps (Figure 3, Table S4), representing the entire range of extraction efficiencies as obtained within the testing of organic chemicals in soil matrices.

Figure 18: Variability of NER fraction for triclosan in Lufa 2.2 (Table A33).



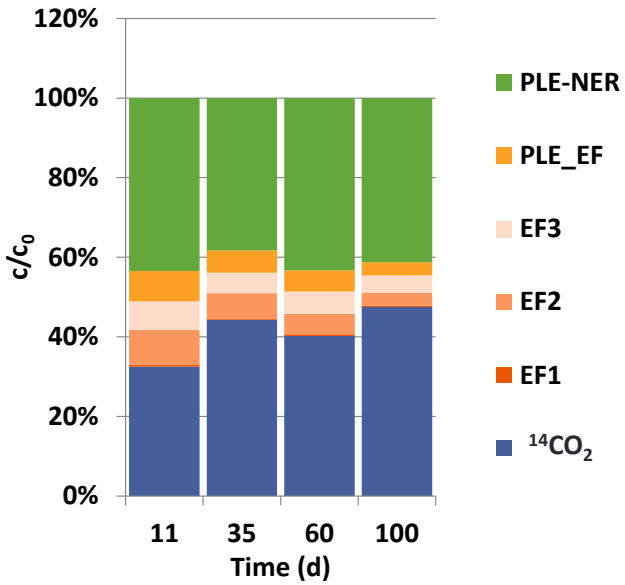
Reference: Federal Institute of Hydrology (2019)

Figure 18 shows the radioactivity extracted by the 4 different extraction steps for Triclosan in Lufa 2.2 at 4 different sampling times.

In the first extraction step (aqueous $CaCl_2$), hardly any radioactivity was extracted from the soil incubated with TCS corresponding with up to 96 % NER. This was expected, since TCS is rather lipophilic (log K_{OW}=4.8/log K_{OC}=4.3) (Dhillon et al., 2015). In the second extraction step (methanol/water), 51 % were extractable after 7 d, whereas less than 8 % were extracted after 100 d. This difference might be explained by ageing effects (Loeffler et al., 2018) and the increasing formation of Me-TCS (Figure S1), which is more lipophilic than TCS (log K_{OW}=5.2 and log K_{OC}=4.6) (Lee, 2015; Lindström et al., 2002). Stopping the extraction procedure after this second extraction step, NER would account for about 89 % of AR. After the third extraction step (methanol/acetone), up to 56 % of AR was extracted, resulting in 35-56 % NER. Finally, PLE released another 6-8 %, corresponding to 28-48 % NER after 100 d of incubation.

These results show, that NER fractions related to TCS varied tremendously between 96 and 28 %, depending on the extraction procedure. A similar behaviour was found for triclosan with the other two soils (Table 8). For FEC and ACT, the variability of NER depending on the extraction procedure was smaller, as the extractability of both compounds was generally lower. NER decreased from 52-60 % after step 1 to 41-52 % after PLE for ^{14}C-FEC and for ^{14}C-ACT from 88-96 % after step 1 to 84-91 % after PLE. Hence, solely 8-11 % AR were extracted by PLE for FEC and 4-5 % for ACT.

Figure 19: Variability in the extent of NER of fenoxycarb in Lufa 2.2(Chapter A4.3.6).



Reference: Federal Institute of Hydrology (2019)

In Table 8 also the variability of NER in dependence on the extraction step is summarised for all compounds and soils. In case of fenoxycarb and acetaminophen the differences are not as significant as they were found for triclosan. For fenoxycarb fractions of 1–4 % of applied radioactivity are released during every extraction step, which is also illustrated for fenoxycarb in Lufa 2.2 in (Figure 19). Thus, the NER fractions after every extraction step vary in a smaller range as for triclosan.

Table 8: Variability of NER in dependence of the extraction procedure (Table A36-38; A61-A63; A80-A82).

Compound	Lufa soil	Time (d)	$^{14}CO_2$ (%)	total radioactivity in soil after 100 d (%)	"NER" after batch extraction step 1/3 (%)	"NER" after batch extraction step 2/3 (%)	"NER" after batch extraction step 3/3 (%)	PLE-EF (%)	"NER" after PLE (= PLE-NER) (%)
TCS	2.2	100	11 ± 0	96 ± 5	96 ± 0	89 ± 0	35 ± 3	6 ± 1	28 ± 3
	2.3	100	21 ± 0	78 ± 2	78 ± 0	73 ± 0	56 ± 1	8 ± 1	48 ± 6
	2.4	100	27 ± 0	75 ± 2	75 ± 0	70 ± 1	35 ± 4	7 ± 2	28 ± 6
FEC	2.2	100	48 ± 3	52 ± 3	52 ± 0	49 ± 0	45 ± 0	3 ± 0	41 ± 4
	2.3	100	43 ± 3	57 ± 3	57 ± 0	54 ± 0	51 ± 0	2 ± 0	49 ± 6
	2.4	100	40 ± 1	60 ± 1	60 ± 0	58 ± 0	55 ± 0	2 ± 0	52 ± 3
ACT	2.2	35	14 ± 0	92 ± 4	91 ± 0	90 ± 0	89 ± 0	3 ± 0	87 ± 11
	2.3	35	18 ± 1	89 ± 5	88 ± 0	88 ± 0	87 ± 0	4 ± 1	84 ± 15
	2.4	35	11 ± 0	96 ± 3	96 ± 0	95 ± 0	95 ± 0	3 ± 0	91 ± 4

Reference: Federal Institute of Hydrology (2019)

For ACT the extractability was even lower, as for FEC. The applied 3SBE released only about 2 % and PLE released between 3 and 4 % of applied radioactivity.

It can be concluded that the percentage of NER observed for a compound is strongly variable with the extraction procedure used. DT_{50} values can be affected directly by the extraction procedure, as a more intense extraction procedures may release a higher proportion of a target compounds and its TPs, leading to increased values for the degradation time, which may be of consequence for the assessment of a compound's persistence. Frequently, the fraction of NER can easily be directed by the experimentalist by applying an extraction procedure "suitable" for purpose and outcome of the study. Therefore, it would be valuable to perform a strong and exhaustive extraction procedure to prevent an overestimation of the NER quantities and to improve the comparability of the results.

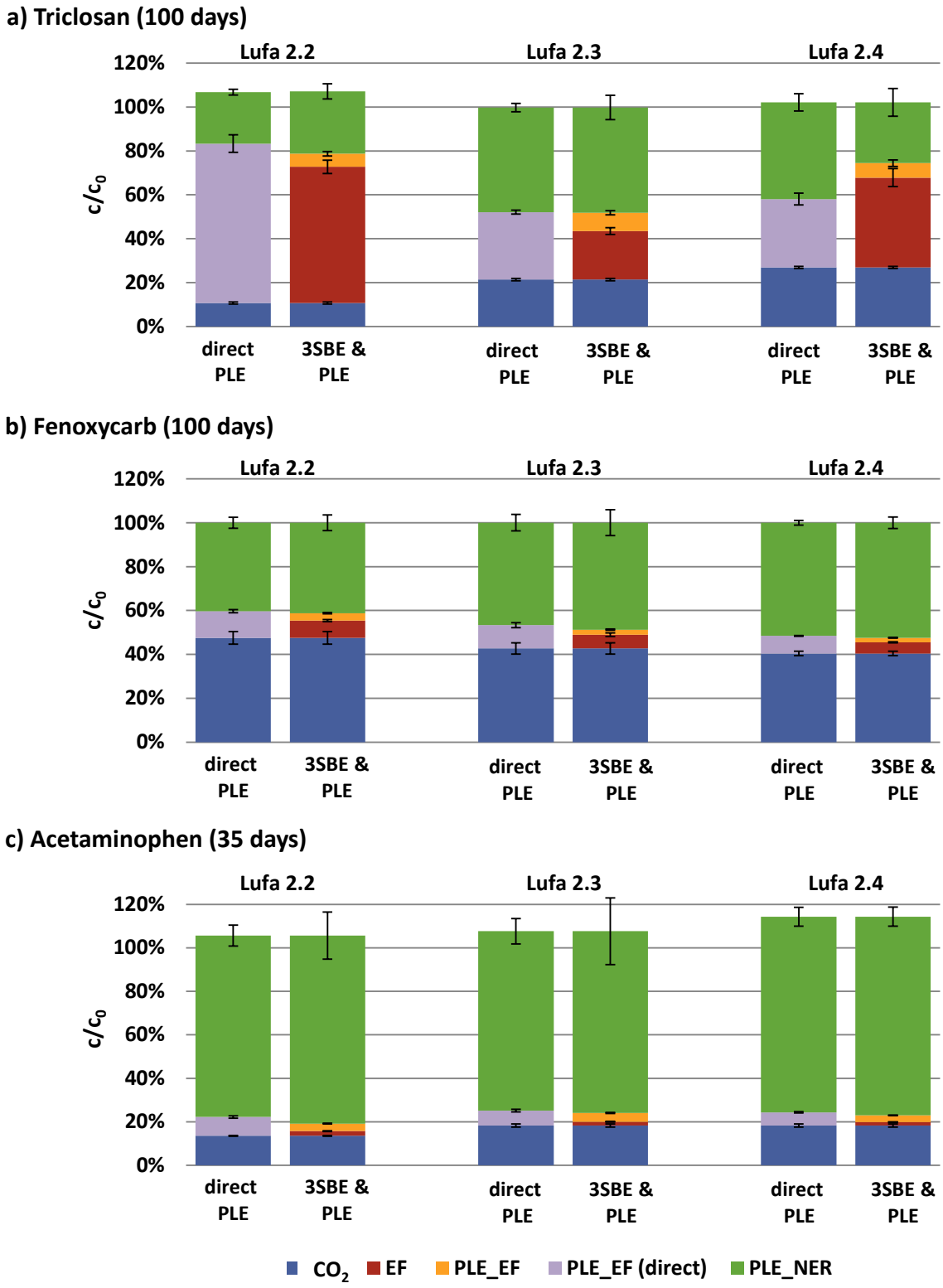
4.3.5 Comparison of direct PLE vs. sequential batch extraction and PLE (3SBE & PLE)

It was shown in our study that the percentage of NER can be highly variable, depending on the extraction procedure used (chapter 4.3.4). This leads to a low comparability of NER data and possibly to an overestimation of NER. PLE with a ternary extraction mixture (methanol/acetone/water) provided a widely exhaustive extraction, excellent extraction efficiencies for a wide range of organic chemicals and a low variability of the analytical data.

The results obtained from sequential batch extraction and consecutive PLE were compared to those using the optimised PLE procedure only (= direct PLE). The results of the direct PLE treatment of the test soils are shown in Figure 20 for each of the three compounds and soils in direct comparison to those of the sequential extraction using 3SBE & PLE.

In the experiment of triclosan with Lufa 2.2, direct PLE released 73 ± 4 % (purple bar) of applied radioactivity, resulting in NER of 23 %. The sequential 3SBE & PLE of the same soil released together 68 ± 4 %, resulting 28 % NER. Considering the statistical uncertainties of both extraction procedures the results were comparable. This was also the case for the other soil/compound combinations, as direct PLE and sequential 3SBE & PLE provided the same proportions of the extractable fraction. Also, the remaining radioactivity in soil after PLE (PLE_NER) showed comparable values. Thus, it can be concluded that direct PLE is a sufficient extraction technique.

Figure 20: Comparison of “direct PLE” with the sequential “3SBE & PLE” (see Tables A42, A67 and A83)



Reference: Federal Institute of Hydrology (2019)

4.3.6 Additional experiments for the characterisation of PLE_NER

After the extraction of incubated soil from the OECD 307 transformation tests with different extraction methodologies of increasing extraction efficiency, further experiments for the characterisation of NER were accomplished following the recommendations by Eschenbach and Oing (Eschenbach and Oing, 2013b). NER-Type 1, the strongly sorbed fraction, was discussed in detail in the previous chapters being accessible with PLE.

In the following sections further experiments for the determination of physically entrapped residues (NER-Type 2) and biogenic NER are discussed.

4.3.6.1 General remarks on physically entrapped NER

The concept of physically entrapped residues in soil assumes that compounds or their TPs are physically entrapped in cavities of soil particles or in soil organic matter (SOM). Thus, the release of those compounds from soil, e.g. via solvent extraction, is inhibited (Cheng et al., 2012; Steinberg et al., 1987). It is assumed that these entrapments are stabilised via polyvalent cations, hydrogen bonds, organic metal-complexes and van der Waals-forces (Calderbank, 1989; Gevao et al., 2000; Kaestner et al., 2014; Steinberg et al., 1987).

However, it should be noted that a precise distinction between sorption and entrapment is hardly possible, as the different mechanisms leading to a formation of NER are often interrelated and the proportion of the entrapped NER fraction is defined operationally.

The release of entrapped organic compounds should be fostered by destroying the stabilising forces mentioned above. Silylation is proposed as one option and was performed in this context within a few studies (Dec et al., 1997b; Haider et al., 2000; Haider et al., 1992; Haider et al., 1993; Wang et al., 2017c). Another approach proposed by Eschenbach and Oing was the addition of complexing agents such as EDTA (Eschenbach et al., 1998). Experiments covering both procedures were conducted within our study and are comprehensively discussed in the following sections.

There are only a limited number of studies describing physically entrapped residues being released and identified after silylation, especially when working with radiotracers. However, in all of these studies the NER were determined after batch or Soxhlet extraction prior to silylation, and thus the quantities of AR remaining in the soils before silylation were comparably higher as for 3SBE&PLE. It may be questioned whether physically entrapped residues are still present in reasonable quantities in the soil matrix after PLE, applying elevated pressure (here 100 bar) and elevated temperatures (here 100°C) (Bester, 2009; Larivière et al., 2016; Nieto et al., 2008; Porschmann and Plugge, 1999; Schantz, 2006; Subedi et al., 2015; Vazquez-Roig and Picó, 2015; Wang et al., 2007). Furthermore, it is likely that PLE changes the molecular environment of compounds sequestered in the soil matrix, affecting molecules bound by van der Waals or hydrogen-bonds.

To our knowledge, there is no clear evidence that physically entrapped residues in soil are still relevant after PLE. For that, further research is necessary.

4.3.6.2 Silylation

The silylation derivatises functional groups such as hydroxy groups into their respective trimethylsilyl ethers. A widely used and very effective silylation agent is trimethylchlorosilane (TMCS), which is added in excess to the soil sample (Dec et al., 1997a; Haider et al., 1992; Haider et al., 1993). The approach of the silylation is to break hydrogen bonds between polar functional groups and to change the hydrophilicity of SOM moieties, resulting in a partial disintegration of the humic substances into smaller fragments, which have been held together in supramolecular aggregates by noncovalent interactions in the original sample (Kaestner et al., 2014). If NER are entrapped in the humic matrix, they are released after silylation, while NER formed by covalent binding remain bound to the fragmented humic matter. These two scenarios can be distinguished, for instance, by size exclusion

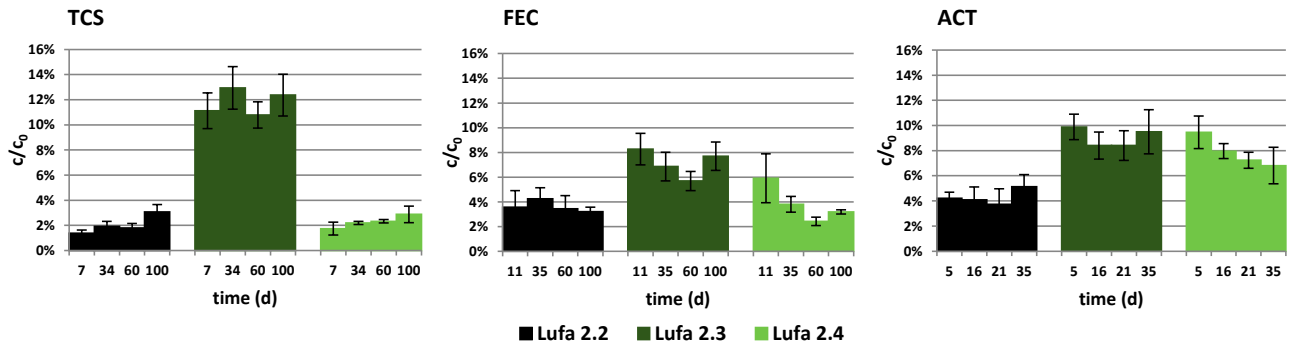
chromatography. In the case of entrapment, the released NER will elute from the matrix according to the molecular size of the parent molecule or the TP. In case of a covalent binding of the labelled parent molecule or TP to a humic substance fragment, the elution time will be according to the size of the fragmented humic matter, typically in the range of a few thousand Da, eluting with shorter retention times than the parent compound or TP. However, this procedure alone does not provide detailed information about the chemical identity of the NER as long as the size fractions are not characterised further.

Results and discussion

For our experiments, acetone was added as solvent to the PLE residues and then TMCS was added. The slurry was shaken overnight (see chapter 4.2.11.1). Afterwards, the released radioactivity in the liquid phase was determined.

Figure 21 displays the proportions of applied radioactivity mobilised by silylation for all compounds, soils and 4 incubation times. For triclosan in Lufa 2.2 and Lufa 2.4 fractions of 3 % were released by silylation, whereas in Lufa 2.3 this amounted to 12 % (A84-A86). The latter is consistent with the observation, that the highest NER formation for TCS occurred in Lufa 2.3 (chapter 4.3.1). Only for Lufa 2.3 and Lufa 2.4 a slight temporal increasing tendency can be observed. An identification and quantification of test substances and their TPs in the silylated extracts was impossible, due to the low quantity of radioactivity released and reasons discussed below.

Figure 21: Release of radioactivity by silylation of pre-extracted soils (3SBE&PLE)



Reference: Federal Institute of Hydrology (2019)

In case of fenoxycarb proportions of 3–8 % of applied radioactivity were released by silylation. Again, the radioactivity released from Lufa 2.3 was slightly higher as compared to the other soils (table A87–A89). Although NER were extensively formed from ACT in soil, silylation of the soils spiked with acetaminophen released only 4 % of applied radioactivity from Lufa 2.2 and about 8 % from Lufa 2.3 and Lufa 2.4, respectively (table A87-89). Significant temporal tendencies were not observed. Since the fractions released by silylation were below 10 % of applied radioactivity for acetaminophen and fenoxycarb, no chemical analysis could be performed due to the limited radioactivity extracted.

4.3.6.3 EDTA-Extraction

Treatment with EDTA was proposed by Eschenbach and Oing (Eschenbach and Oing, 2013b) as an alternative method for the determination of physically entrapped radioactivity in soil (Achtnich et al., 1999b; Eschenbach et al., 1998; Weiss et al., 2004). Cations like Ca^{2+} contribute to the stabilization of the soil matrix. By complexing those cations, e.g. by addition of EDTA, the soil matrix may be destabilised leading to a release of compounds being entrapped in cavities in the soil matrix.

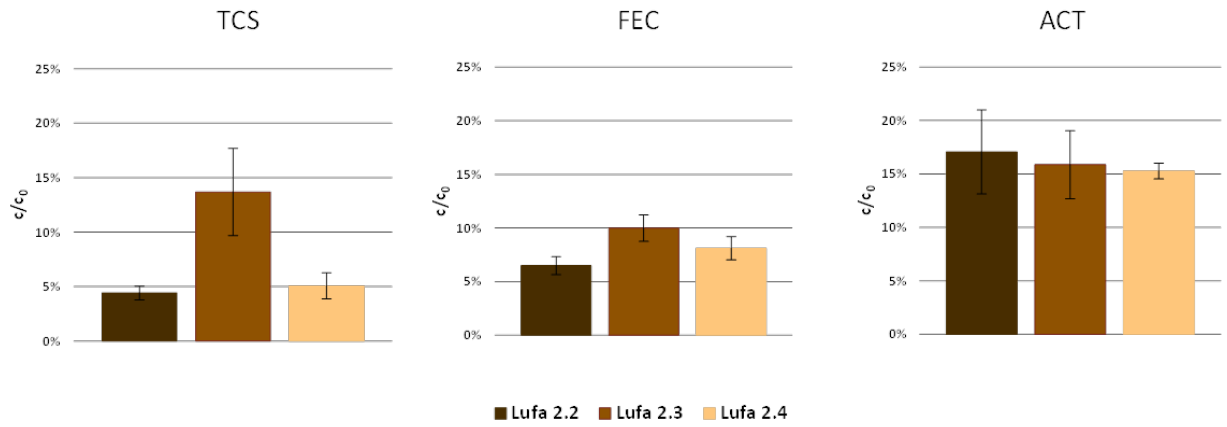
Results and discussion

To study the effect of EDTA on the release of incubated triclosan, fenoxycarb and acetaminophen, the pre-extracted soil (3SBE&PLE) was mixed with EDTA solution and shaken overnight as described above (chapter 4.2.11.2). This treatment was performed for all soils and compounds after a total incubation time of 100 d and 35 d, respectively.

Figure 22 illustrates the proportions of applied radioactivity being released by EDTA extraction. For triclosan fractions of about 5 % were released from Lufa 2.2 and Lufa 2.4, whereas for Lufa 2.3 14 % were released (Table A93).

For fenoxycarb proportions of 6–10 % were released from all soils by EDTA treatment (Table A91). The highest amounts released were found for acetaminophen, for which 15–17 % of the applied radioactivity were released from the soils (Table A94).

Figure 22: Results of the EDTA extraction[7]



Reference: Federal Institute of Hydrology (2019)

Chemical analysis of EDTA extracts were conducted for triclosan in Lufa 2.3 only. For that, the aqueous extract containing EDTA was extracted using liquid-liquid extraction (LLE) with heptane followed by evaporation, re-uptake in methanol/water and radio-HPLC analysis as described before. While it was shown that TCS and non-polar TPs such as Me-TCS distribute primarily into the heptane phase during LLE, no significant quantities of radioactivity were found in the heptane phase after EDTA extraction. Therefore, it can be concluded that neither triclosan nor non-polar TPs such as methyl-triclosan were released by EDTA extraction. Thus, it is likely that polar TPs, possibly of biogenic origin, were extracted by EDTA extraction.

Chemical analysis of the EDTA extracts from acetaminophen was not performed since no appropriate analytical method was established. However, it is known from literature that acetaminophen is quantitatively degraded in soil within hours (Li et al., 2014).

EDTA extraction mobilised further radioactivity from the incubated and pre-extracted soils. However, from the experiments conducted here there is no indication that significant quantities of the parent compounds or their major TPs were released by EDTA extraction.

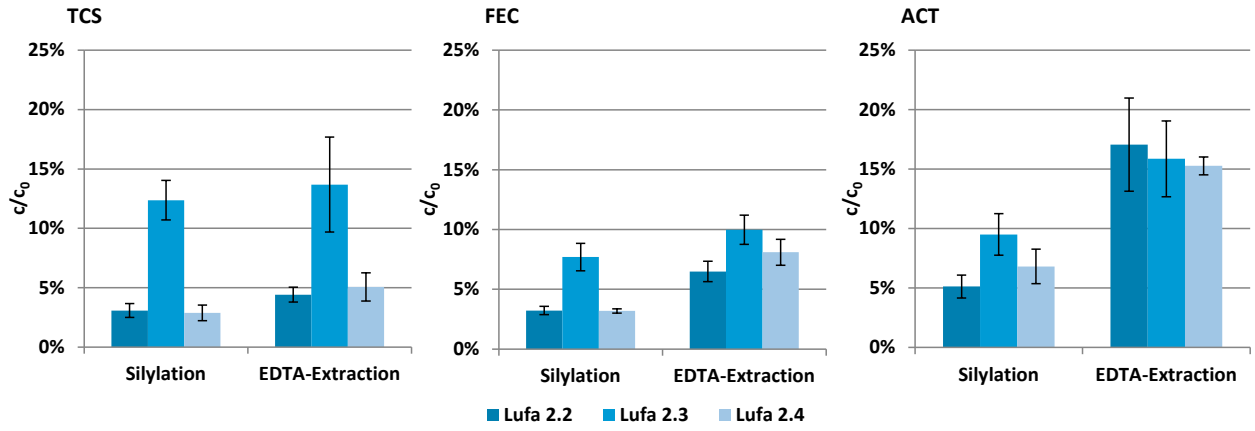
[7] The bars represent the radioactivity mobilized during EDTA extraction in relation to the applied radioactivity at test start.

4.3.6.4 Comparison of silylation and EDTA-Extraction – Advantages and Drawbacks

Comparing the results for EDTA extraction with those for silylation, it can overall be observed that the percentage released by EDTA extraction is higher as by silylation (Figure 23).

Comparable quantities were found for triclosan showing that in Lufa 2.3 significantly more radioactivity was released by both treatments in comparison to the other soils. For fenoxycarb the EDTA extraction yielded slightly but significantly more radioactivity than silylation. However, the most noticeable differences were observed for acetaminophen, where significantly more radioactivity was mobilised by the EDTA extraction than by silylation, possibly due to the most extensive formation of NER of all compounds investigated.

Figure 23: Comparison of silylation and EDTA extraction[8]



Reference: Federal Institute of Hydrology (2019)

Significant amounts of radioactivity are released from the soil by silylation as well as by EDTA extraction. However, there is no indication that significant quantities of the parent compounds or their major TPs were released by EDTA extraction. Also, other authors found no increase of extractable parent or TPs using EDTA (Achtnich et al., 1999b; Eschenbach et al., 1998; Weiss et al., 2004).

In an exemplary experiment, soil samples subjected to EDTA extraction were subsequently silylated and released small quantities of radioactivity. It may be assumed that EDTA addition and silylation are releasing different fractions of radioactivity, as the release mechanisms are totally different, and in both cases not fully understood. While silylation primarily affects the SOM by chemical modification, EDTA is complexing cations in the soil.

A major drawback of silylation for chemical analysis in radiotracer studies is that parent molecules and their transformations products are likely to be silylated as well, as long as they are bearing functional groups prone for silylation. Since silylation is not always quantitative in these complex soil mixtures, a variety of differently silylated parent compounds and transformation products can be formed, impeding their chromatographic separation, identification and quantification. Furthermore, it has to be noted that the silylated matrix itself is very complex and difficult to analyse. Silylated compounds are prone to hydrolysis, which is of consequence when analyzing these using reversed phase HPLC, as frequently done in radiotracer analysis. To our impression it is extremely challenging to attain reproducible and comparable results if target compounds and their TPs are foreseen to be quantified. Furthermore, it would be very challenging to interpret the released radioactivity, since silylation does not differentiate between physically entrapped and biogenic transformation products.

[8] The bars represent the radioactivity mobilized in relation to the applied radioactivity at test start.

It is worth mentioning, that EDTA is chemically milder in comparison to silylation. A major benefit is that the chemical analysis of EDTA extracts should be feasible in most cases, while it is rather challenging after silylation. Quantitative analysis after silylation using radiotracer techniques might be possible at high spiking levels, however, quantitation is still a problem due to the high variety of differently silylated compounds (parent and transformation products). It cannot be excluded that EDTA extraction and silylation are releasing different substances/fractions as the release mechanism is different. However, due the much easier performance and the higher efficiencies the EDTA extraction seems favourable, especially when chemical analyses of entrapped residues are conducted.

4.3.6.5 Biogenic NER

Non-extractable residues from organic chemicals pollutants, which are transformed into biomolecules (e.g. proteins, fatty acids, nucleic acids, sugars, amino sugars) and are incorporated into the biomass, are defined as biogenic NER. For soil microbiota, these compounds and their TPs can pose as carbon source or may be used as energy source. Which of these processes dominates depends on various parameters such as the properties of the target compounds, their concentrations, the microbiological community present, and soil characteristics (e.g. SOM/DOC) (Kaestner et al., 2014).

Residues which are transformed into biomass are explicitly excluded from non-extractable residues in the environmental risk assessment (Calderbank, 1989; Roberts, 1984). Since the compounds are transformed into biomolecules, no possible environmental risk is anticipated. A possible risk for the environment from extractable and non-extractable residues is only expectable from the parent compound and its transformation products.

In scientific literature and the study presented by Eschenbach and Oing only a limited number of methods are described for the determination of those biogenic residues (Brock et al., 2017; Eschenbach and Oing, 2013b; Nowak et al., 2013; Poßberg et al., 2016; Trapp et al., 2017; Wang et al., 2017a; Wang et al., 2016; Wang et al., 2017b). The aim of those studies was always to trace the labels implemented in the applied compounds up to biomolecules formed within these experiments. These biomolecules can be proteins, fatty acids, nucleic acids, sugars, amino sugars and others. Mostly, the proteins, respectively amino acids are analysed since they represent the main constituent in bacteria.

For ^{13}C labelled test compounds numerous publications are available from Kästner et al. addressing the conversion of xenobiotics to biogenic NER (Brock et al., 2017; Girardi et al., 2011; Kaestner, 2000; Kaestner et al., 2016; Kaestner et al., 2014; Kaestner et al., 1999; Nowak et al., 2013; Nowak et al., 2011; Nowak et al., 2020; Wang et al., 2016; Wang et al., 2017b). However, publications dealing with the formation of biogenic residues from ^{14}C labelled compounds are rare.

Only publications from Possberg et al. and Claßen et al. investigated the formation of biogenic residues in soil originating from radiolabelled compounds (Claßen et al., 2019; Poßberg et al., 2016). Poßberg et al. traced the ^{14}C label of bromoxynil after a conversion into amino acids with a laborious procedure. Briefly, after spiking and incubation the soil was Soxhlet extracted with methanol obtaining NER containing soil. This pre-extracted soil was heated in 6 M HCl achieving the complete hydrolysis of the proteins down to the amino acids. After concentration and clean-up, the protein hydrolysate was separated by thin layer chromatography. Selected spots where then scratched off the TLC plate, analysed and quantified by means of LC/MS, Radio-HPLC and GC/MS. Based on these results and using certain assumptions the proportion of applied radioactivity in the protein fraction was extrapolated to the biogenic NER.

However, this procedure is very laborious and requires, besides special analytical instruments, an extensive method development and high specific activities of the ^{14}C-radiotracers. Within the project presented herein it was not possible to establish the entire analytical methodology described above.

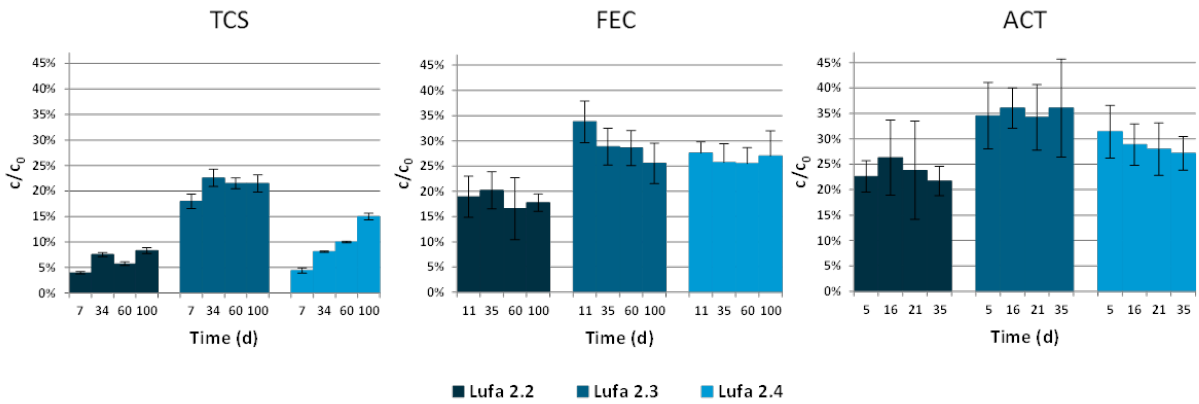
Nevertheless, the first step of the procedure of Possberg et al., the HCl hydrolysis of soils was carried out in this study (Poßberg et al., 2016).

Acidic hydrolysis of proteins in the bio-NER containing soils results an aqueous hydrolysate which can be analysed for radioactivity and amino acids originating from the bio-NER. Hence, pre-extracted soil (3SBE&PLE) was treated with boiling 6 M HCl to obtain a rough impression whether bio-NER had been formed from the three ^{14}C-labelled compounds applied (Poßberg et al., 2016) (chapter 4.2.11.3). However, non-biogenic NER might also be released by applying the HCl treatment.

Results and discussion

The pre-extracted soils were treated with hydrochloric acid to hydrolyse all proteins down to their consisting amino acids. While proteins of the bio-NER are widely inseparably associated with the soil matrix proteins, the hydrolysis by HCl treatment provided an aqueous hydrolysate. Radioactivity recovered in the hydrolysate is shown in Figure 24.

Figure 24: Results of the HCl hydrolysis and following MeOH extraction



Reference: Federal Institute of Hydrology (2019)

For triclosan proportions of 4–23 % of applied radioactivity were released within this step. Again, significantly more radioactivity was released from Lufa 2.3 soil due to its high NER content. With increasing incubation time more radioactivity was released from Lufa 2.4 (Table A96–A98).

In case of fenoxycarb proportions of 17–34 % of applied radioactivity were mobilised over all test soils and sampling times (Table A99–A101). Similar results were obtained for acetaminophen, where 22–36 % of applied radioactivity was released (Table A102–A104). For fenoxycarb and acetaminophen, no significant time dependency was observable.

The radioactivity, which was mobilised by HCl hydrolysis was probably released due to different mechanisms. In addition to the hydrolysis of any types of proteins in the soil, also other residues may be released (Poßberg et al., 2016). Nevertheless, to our interpretation these results may still indicate that relevant quantities of bioNER were formed in the course of the incubation experiments.

Especially for fenoxycarb and acetaminophen, which were rapidly transformed and significantly mineralised and poorly extracted by 3SBE&PLE, it is likely that their breakdown products were significantly incorporated into microbial biomass. In comparison, for fenoxycarb and acetaminophen HCl treatment released significantly more radioactivity from the soil, than silylation or EDTA extraction, while the amounts released for triclosan were similar. However, neither TCS, FEC nor their main transformation products were detected in the hydrolysates, applying LLE. Thus, it is likely that biogenic compounds such as amino acids are extracted by HCl hydroloysis.

Nevertheless, this hypothesis has yet to be confirmed by further analysis (Table 9), which was not within the scope of our study.

For a detailed speciation of the various types of NER, additional analyses are necessary. Future research should be targeted on the characterization of NER (e.g. after EDTA extraction, silylation or HCl-treatment) with a special focus to possible biological effects.

Table 9: Summary of the fraction remaining in soil after incubation period, 3SBE&PLE and silylation, EDTA extraction or HCl hydrolysis[9]

Compound	Soil type	Time (d)	PLE-NER (%)	Remaining radioactivity in soil after 3SBE, PLE and Silylation (%)	EDTA extraction (%)	HCl hydrolysis (%)
Triclosan	Lufa 2.2	100	28 ± 3	25 ± 1	24 ± 1	20 ± 1
	Lufa 2.3	100	48 ± 6	36 ± 2	34 ± 4	26 ± 3
	Lufa 2.4	100	28 ± 6	25 ± 1	23 ± 1	13 ± 4
Fenoxycarb	Lufa 2.2	100	41 ± 4	38 ± 0	35 ± 1	23 ± 2
	Lufa 2.3	100	49 ± 6	41 ± 1	39 ± 1	23 ± 4
	Lufa 2.4	100	52 ± 3	49 ± 0	44 ± 1	25 ± 5
Acetaminophen	Lufa 2.2	35	87 ± 11	81 ± 1	69 ± 4	65 ± 3
	Lufa 2.3	35	84 ± 15	74 ± 2	68 ± 3	48 ± 10
	Lufa 2.4	35	91 ± 4	85 ± 1	76 ± 1	64 ± 3

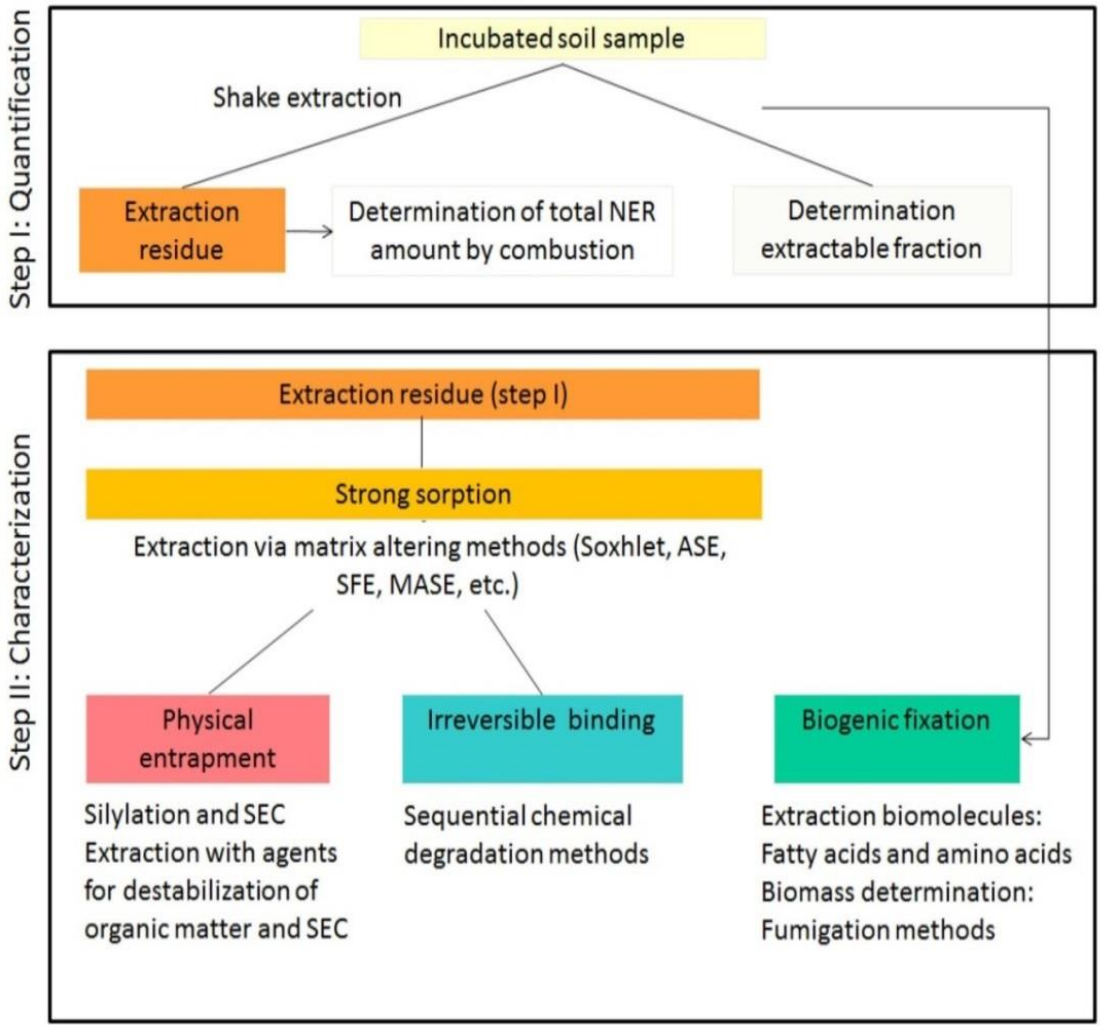
Reference: Federal Institute of Hydrology (2019)

[9] All values are related to applied radioactivity at test start.

5 Revision of the extraction scheme by Eschenbach and Oing and recommendations for a new NER-extraction scheme

The sequential extraction scheme of Eschenbach and Oing (Figure 25 and Table 10) based on a literature survey (Eschenbach and Oing, 2013b), classifying NER into four types.

Figure 25: Sequential extraction scheme for the characterisation of non-extractable residues by Eschenbach and Oing



Reference: (Eschenbach and Oing, 2013b)

NER of type 1 comprise residues which are associated with soil by strong sorption, while NER of type 2 are residues which are physically entrapped in the soil matrix and NER of type 3 are irreversibly bound to the soil matrix by covalent bonding.

NER of type 1-3 are defined by the nature of the association of a parent compound or its TPs to the soil matrix.

In contrast, NER of type 4 are defined as all biomolecules formed after biogenic fixation, where xenobiotics and their transformation products are used for the build-up of biomass. It should be noted that NER of type 4 are contained in the fractions corresponding to the NER types 1-3, as the biogenic NER can be associated with the soil in the same way as other organic chemicals (sorption, entrapment, covalent binding).

Table 10: Classification of NER types according to Eschenbach and Oing (Eschenbach and Oing, 2013b)

NER type	NER mechanism	Analytical approach
1	Strong sorption	PLE, Soxhlet, SFE, MASE
2	Physical entrapment	Silylation, EDTA extraction
3	Irreversible (covalent) binding	Sequential chemical degradation
4	Biogenic fixation	Extraction of biomolecules

Reference: Federal Institute of Hydrology (2019)

In general, the discussion of NER suffers from the low comparability of NER data. When extraction techniques of low extraction intensity (e.g. batch extraction) are used for the determination of the extractable fraction, a high variability of the NER data can be expected (chapter 4.3.4) for the corresponding fraction of non-extractable residues. It should be emphasised that NER might be significantly overdetermined, when the extraction efficiency is low.

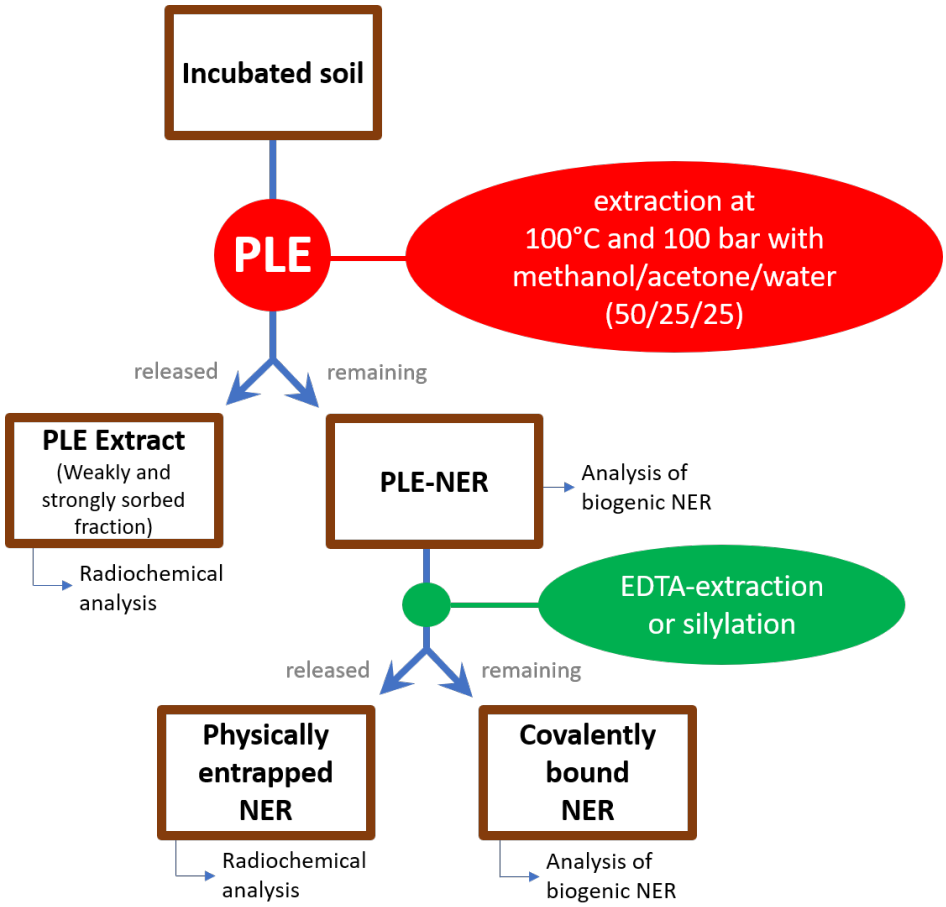
A main task of our study was to develop a general procedure for the quantification and characterisation of non-extractable residues (NER) in soils. As a consequence of the low comparability of NER data resulting from batch extraction (see chapter 4.3.4) a revised extraction scheme for non-extractable residues was developed (Figure 26).

PLE was applied in our widely universal extraction procedure, providing elevated extraction efficiencies and a low variability using a ternary extraction agent (see chapter 3.4). The PLE extraction provides extraction efficiencies mostly higher, than other extraction techniques for a variety of analytes (Bester, 2009; Porschmann and Plugge, 1999; Wang et al., 2007) and can be used either as the only extraction step or within a sequential extraction procedure.

The extracts released by PLE (**PLE extracts**) contain **all extractable compounds** including weakly and strongly sorbed fractions (Figure 26). We would like to note, that this includes proportions of those residues, which would have been considered entrapped residues, when using less efficient extraction procedures such as batch extraction. The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) even considers the intensity of PLE as very high, being short of digestion or combustion (ECETOC, 2013a).

It cannot be ruled out that biogenic transformation products (TPs) are mobilised by PLE as well, e.g. by destruction of microbial cells. However, in radiochemical analysis, these transformation products are likely to be described as unknown TPs.

Figure 26: Revised extraction scheme proposed by the BfG.



Reference: Federal Institute of Hydrology (2019)

The residues in the soil after PLE were named **PLE_NER** and consist of entrapped residues remaining in the soil and residues covalently bound to the soil including those of biogenic origin.

The application of **PLE_NER** for quantification and characterization of NER allows for a significantly better comparability of NER data, due to the widely exhaustive extraction by PLE.

PLE_NER was then used for a characterisation of entrapped residues which can be accomplished in the first step preferably by EDTA extraction, destabilizing the soil matrix by complexing cations as Ca^{2+} (see chapter 4.3.6.3). Alternatively, silylation can be used (see chapter 4.3.6.4) when focussing on the SOM (Kaestner et al., 2014; Schaeffer et al., 2018). The entrapped fraction can be characterised further by size exclusion chromatography (Kaestner et al., 2014) and radiochemical analysis.

Residues remaining from EDTA extraction can be used for the quantification and characterisation of the covalently bound soil fraction including the contained biogenic NER (Eschenbach and Oing, 2013b).

It should be noted, that the **PLE_NER** can be used to characterise and quantify the biogenic NER within.

Besides the results of our study presented herein, several authors presented similar classification schemes for NER (Eschenbach and Oing, 2013b; Kaestner et al., 2018; Schaeffer et al., 2018).

The main difference between the approaches is (Table 11), that while Eschenbach and Oing (Eschenbach and Oing, 2013b) referred to four NER types including the strongly sorbed fraction (Type 1), only three NER types were defined by Kästner et al. and Schäffer et al. (Kaestner et al., 2018; Schaeffer et al., 2018), **not** accounting the extractable portion of the strongly sorbed fraction as NER.

Their NER Type I subsumes the non-extractable portion of the strongly sorbed fraction and the physically entrapped residues, addressing these both as sequestered (Kaestner et al., 2018; Schaeffer et al., 2018).

Due to the high extraction efficiency obtained by the widely universal PLE procedure presented within this work, the PLE extraction applied can be assumed to be exhaustive. Hence, the strongly sorbed fraction is released completely by PLE. As a consequence, the residue remaining after PLE (=**PLE_NER**) merely contains the physically entrapped fraction and the fraction covalently bound to the soil (Table 11, columns *Loeffler and Ternes*).

Table 11: Boundaries and interrelation of NER-Types and Fractions

Eschenbach and Oing 2013		Kästner and Schäffer 2018		Loeffler and Ternes	
Type	NER Characteristics	Type	NER Characteristics	Fraction	NER Characteristics
1	Strongly sorbed NER	-	Strongly sorbed (extractable[1])	PLE extract	Strongly sorbed (extractable[3])
		I	Strongly sorbed (non-extractable[2]) and physically entrapped NER (both = sequestered NER)		
2	Physically entrapped NER			PLE-NER	Physically entrapped NER
3	Irreversibly bound/ covalently bound NER	II	Covalently bound		Covalently bound NER
4	Biogenically fixated NER	III	Incorporation into biomass	(Biogenic NER are not considered here as an individual fraction)	

[1] Released by intense extraction procedures (e.g. PLE) and hence not accounted as NER

[2] Not released by intense extraction procedures (e.g. PLE) and hence accounted as NER

[3] Released by PLE and hence not accounted as NER

Reference: Federal Institute of Hydrology (2021)

Similar to Eschenbach and Oing (Eschenbach and Oing, 2013b), Schaeffer et al. and Kaestner et al. (Kaestner et al., 2018; Schaeffer et al., 2018) defined the biogenic NER as an additional NER type (Type III).

It must be emphasised that biogenic residues may occur in all soil or sediment fractions (e.g. as dissolved, weakly sorbed, strongly sorbed, physically entrapped or covalently bound). Hence, biogenic NER were not considered here as an individual analytical fraction (Table 11, columns *Loeffler and Ternes*).

However, the consideration of the biogenic NER is an important issue from the regulatory point of view.

For a consistent discussion of NER it is recommended to use the term biogenic NER only for those fractions which are non-extractable. In this sense biogenic NER should be present mainly as physically entrapped residues which are considered mostly reversibly bound and as residues covalently bound to the soil (e.g. to SOM) which are considered mostly irreversibly bound.

The entirety of biogenic residues of an organic chemical may be considered as safe sink, within the environmental risk assessment (Eschenbach and Oing, 2013a; Schaeffer et al., 2018).

However, addressing fractions inadequately, e.g. dissolved or weakly sorbed biogenic residues as biogenic NER, will lead to ambiguity and confusion in the discussion.

6 Conclusions and Outlook

The comparability of NER results in soil is a challenge when using non-exhaustive extraction methods, such as batch extraction etc., as portions of the target compounds analysed are likely to remain in the soil matrix after extraction. For investigating the fate of an organic chemical, all fractions of this compound have to be considered independently whether these may be present as the original compound, as transformation products or whether these may be associated with the soil as NER by different mechanisms, e.g. sorption, entrapment. Following this conservative approach, a widely general procedure for the analysis of NER was developed applying PLE using a ternary extraction solvent, which provides elevated extraction efficiencies and a low variability. Additional experiments using silylation, EDTA-extraction and HCl-treatment indicated, that NER after PLE (**PLE_NER**) are widely restricted to those proportions of a compound which are covalently and thus irreversibly bound to the soil or incorporated as biogenic molecules. It can be assumed that the observations for the analysis of NER in soils are also applicable for sediments, as these matrices are widely similar, regarding their analysis.

In the regulation of organic substances, NER need to be more integrated into the environmental risk assessment as they can be crucial for the authorization decision. In the past, NER have been widely neglected as only the DT_{50} (primary degradation) of parent compound and transformation products in soil and water/sediment systems (OECD guidelines TG 307, 308, 309) was considered. The ECHA R.11, guideline highlights the importance of NER for the assessment of persistence when testing transformations in water/sediment, water or soil systems (ECHA, June 2017). For this, a harmonised concept to consider potentially remobilisable NER in the framework of persistence assessment (e.g. PBT, vPvB, POP) is required, which can be based on the PLE procedure developed.

A conceivable solution might be to determine the parent compound as well as its transformation products in the extracts of the PLE and in extracts obtained by additional treatments with EDTA-extraction or silylation (entrapped residues). The fraction released by these additional treatments represents the potentially remobilisable NER fraction. For the assessment of the persistence, DT_{50}-values of the parent compound and the transformation products are calculated considering the sum of each compound over all released fractions.

If biogenic residues occur in a certain fraction, the proportions of the biogenic residues should be omitted in these calculations, as the entirety of biogenic residues of an organic chemical may be considered as safe sink (Eschenbach and Oing, 2013a; Schaeffer et al., 2018).

Alternatively, a new trigger for the potentially remobilisable fraction of the NER for the persistence assessment might be established combined with the determined degradation half-lives.

Hence, it is recommended to use the universal PLE procedure presented herein as a standard in environmental fate testing of organic chemicals for regulatory purposes to increase the comparability of NER data. In the presented experiments EDTA extraction of **PLE_NER** released higher quantities of radioactivity than silylation. Future research is necessary to fully elucidate the mechanisms of physical entrapment and effects of procedures such as silylation or EDTA-extraction used in the release of physically entrapped residues.

6.1 Index of Annex

Table of Contents

7 References

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